IgE in Non-Atopic Asthma

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IgE in Non-Atopic Asthma

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I dedicate this thesis to my beloved wife, Preethy, for being on my side & encouraging me throughout; and my supervisor Prof Corrigan for the constant support and guidance. I wouldn't have been able to complete this work without their support.

Abstract

Introduction

Previous studies suggest that IgE switching and synthesis occur in the bronchial mucosa of "non-atopic" asthmatics, suggesting a role for IgE in asthma regardless of conventional atopic status. To investigate this, I addressed the following hypotheses: (1) allergen-specific IgE is manufactured in the bronchial mucosa of "non-atopic" asthmatics but does not appear in the periphery; (2) naturally occurring IgE-specific IgG-autoantibodies may involve IgE in asthma pathogenesis in an allergen-independent manner; (3) anti-IgE therapy improves lung function and bronchial mucosal inflammation in non-atopic asthmatics *in vivo*.

Methods

(1) ImmunoCAP and ImmunoCAP ISAC microarrays were used to detect, quantify and specify IgE in the bronchial mucosa of both atopic and non-atopic asthmatics; (2) bespoke assays were developed to detect and quantify IgE-specific IgG autoantibodies in the sera of atopic and non-atopic asthmatics, and positive sera examined for their ability to activate IgE-sensitised human blood basophils in the presence and absence of allergen using a basophil activation test, and to inhibit allergen binding to specific IgE on a rat basophilic cell line stably expressing human FcεRI; (3) a randomised, double-blind, proof of concept study was performed to address the hypothesis that omalizumab therapy improves lung function and reduces bronchial mucosal IgE expressing cells (utilising immunohistochemistry) in a group of severe, non-atopic asthmatics.

Results

I found that (1) although the median total IgE concentration in bronchial mucosal homogenates was significantly elevated in both non-atopic and the atopic asthmatics

compared with controls, allergen component-specific IgE species were detectable only in the atopic, and not the non-atopic asthmatics; (2) IgG autoantibodies binding to both free and FcεRI-bound IgE were detectable in patients with atopic and non-atopic asthma as well as controls: some were able to activate IgE-sensitised basophils, whereas others inhibited allergen-induced basophil activation, at least partly by inhibiting binding of IgE to specific allergen; (3) omalizumab, but not placebo therapy of a group if non-atopic asthmatics for 20 weeks was associated with significant improvement in absolute and % predicted FEV_1 despite substantial reduction of existing anti-asthma treatment, along with a significant reduction in the median total IgE^+ cells ($p<0.001$) in the bronchial mucosa.

Conclusions

IgE is elevated in the bronchial mucosa of asthmatics termed 'non-atopic' by conventional criteria. Although not allergen-specific, it may play a role in the pathogenesis of bronchial mucosal inflammation in asthma, for example as a result of the effects of auto-anti-IgE antibodies. Blockade of IgE binding to its receptors has the propensity to improve lung function in non-atopic asthmatics.

Statement

The majority of the clinical work presented in this thesis was performed by me with great assistance from the departmental clinical research team. This included participant recruitment, clinical interventions including bronchoscopy, obtaining samples, management of the clinical trial, file maintenance etc. Dr Cailong Fang and Dr Mohammed Shamji guided and assisted me in completing the ImmunoCAP and ISAC experiments. Immunohistochemical and immunofluorescence experiments were performed by Dr Yih-Chih Chan and Miss Celine Wu. Dr Yih-Chih Chan also designed and performed the IgE autoantibody experiments and basophil activation assays. I participated in, and contributed to the laboratory experiments wherever I could.

Acknowledgement

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Abbreviations

AA	Atopic asthma
ABPM	Allergic Bronchopulmonary Mycoses
ACD	Asthma Control Diary
ACQ	Asthma Control Questionnaire
AERD	Aspirin Exacerbated Respiratory Disease
ANA	Anti-nuclear antibody
APAAP	Alkaline phosphatase anti-alkaline phosphatase
APC	Antigen presenting cell
AQLQ	Asthma quality of life questionnaire
BAL	Bronchoalveolar lavage
BAT	Basophil activation test
BMK-13	Barkans Moqbel Kay-13 (Antibody against eosinophilic major basic protein)
CD	Cluster of differentiation
CFTR	Cystic Fibrosis Transmembrane conductance Regulator
CHAPS	3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate
cDNA	Complementary DNA
CS	Corticosteroids
DC	Dendritic cell
DMSO	Dimethyl sulphoxide
EDTA	Ethylene diamine tetra acetic acid
EliA	Enzyme linked immunoassay
ELISA	Enzyme linked immunosorbent assay
FCS	Foetal calf serum

FEV	Forced expiratory volume
GM-CSF	Granulocyte macrophage colony-stimulating factor
HDM	House dust mite
HRP	Horse radish peroxidase
ICAM	Intercellular adhesion molecule
IDM	Information data manager
IgE	Immunoglobulin E
IHC	Immunohistochemistry
IL	Interleukin
γ-IFN	Interferon-γ
ISAC	Immuno solid phase allergen chip
ISU	ISAC standardized unit
LT	Leukotriene
MIA	Microarray image analysis software
MP	Milk powder
MWCO	Molecular weight cut off
NAA	Non-atopic asthma
NAC	Non-atopic controls
ng	Nanogram
NO	Nitric Oxide
OCT	Optimal cutting temperature compound
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with Tween 20
PCR	Polymerase chain reaction

PEFR	Peak expiratory flow rate
PFA	Paraformaldehyde
pg.	Picogram
RT	Room temperature
SBM	Sub-epithelial Basement Membrane
SEA	*Staphylococcus* enterotoxin-A
SEB	*Staphylococcus* enterotoxin-B
SEC	*Staphylococcus* enterotoxin-C
SED	*Staphylococcus* enterotoxin-D
SEE	*Staphylococcus* enterotoxin-E
SmPC	Summary of Product Characteristics
SOP	Standard operating procedure
SPT	Skin prick test
TRIS	Tris (hydroxymethyl) amino methane
TSST-1	Toxic shock syndrome toxin-1
TMB	3, 3′, 5, 5′-Tetramethylbenzidine
TNF-α	Tumour necrosis factor-α
TSLP	Thymic stromal lymphopoietin
VEGF	Vascular endothelial growth factor
WHO	World Health Organization

Chapter 1: General Introduction

1. General Introduction

Asthma is a chronic disorder of the airways characterised by variable and recurring symptoms, airflow obstruction, bronchial hyperresponsiveness and underlying inflammation[1]. It is a leading cause of suffering, affecting about 300 million people worldwide. Asthma is the most common chronic disease among children[2]. In the UK, over 5.2 million people are affected which include 1.1 million children[3]. On average there is one hospital admission for asthma every 7 minutes and one death every 7 hours. Although existing drugs control the disease in most patients, in many thousands they do not, leaving them prey to severe daily symptoms and unexpected exacerbations, which make their lives a misery. These patients need new treatments urgently.

1.1 Asthma Classification

Asthma may be classified in many ways: clinically on the basis of various parameters including the atopic status of the subject, the degree of airways obstruction or the nature of the trigger precipitating an attack. The classification of asthma is complicated by the multidimensional nature of the disease. Thus, the term asthma, like 'arthritis', equates to a definition of grouped clinical and physiological characteristics[4]. Fig 1.1 is a schematic representation of the term 'asthma'.

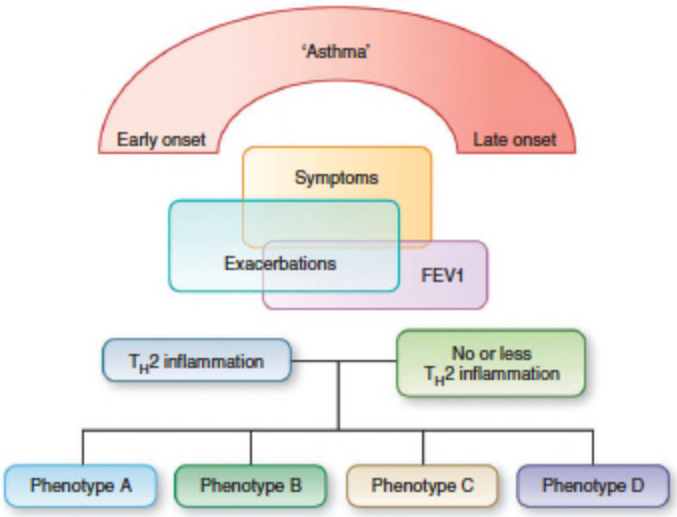


Fig 1.1: Schematic representation of the umbrella term 'asthma'. The key clinical features of severity (lung function, symptoms and exacerbations), inflammatory characteristics (particularly Th2 immunity) and their division into associated phenotypes are shown. However, these phenotypes have not yet been fully characterized[4]

1.1.1 The concept of Asthma Clusters and Clinical Asthma Phenotypes

The most recent approach to asthma classification is to define clusters, phenotypes and endotypes of disease with a view to delineating the so-called "personalised management plan" in the era of novel treatment strategies including biologics. Cluster analysis has been used in an attempt to identify phenotypic subgroups of disease based on distinct patient and clinical disease characteristics[5]. As this is classification system is still evolving and in its early stages, several groups have proposed asthma cluster "phenotypes". For example, the severe asthma group from Leicester proposed 3 clusters emerging from their population data set of milder asthmatics managed largely in primary care: i) early onset atopic, ii) obese non-eosinophilic, iii) benign asthma; and 4 clusters of more severe patients typically managed in secondary care: i) early onset atopic, ii) obese non-eosinophilic, iii) early symptom predominant and iv) inflammation predominant (Fig 1.2). Interestingly, atopic status was not identified as a

significant discriminator influencing cluster membership in any of these clusters in either primary or secondary care, although the prevalence of atopy significantly differed between the clusters.

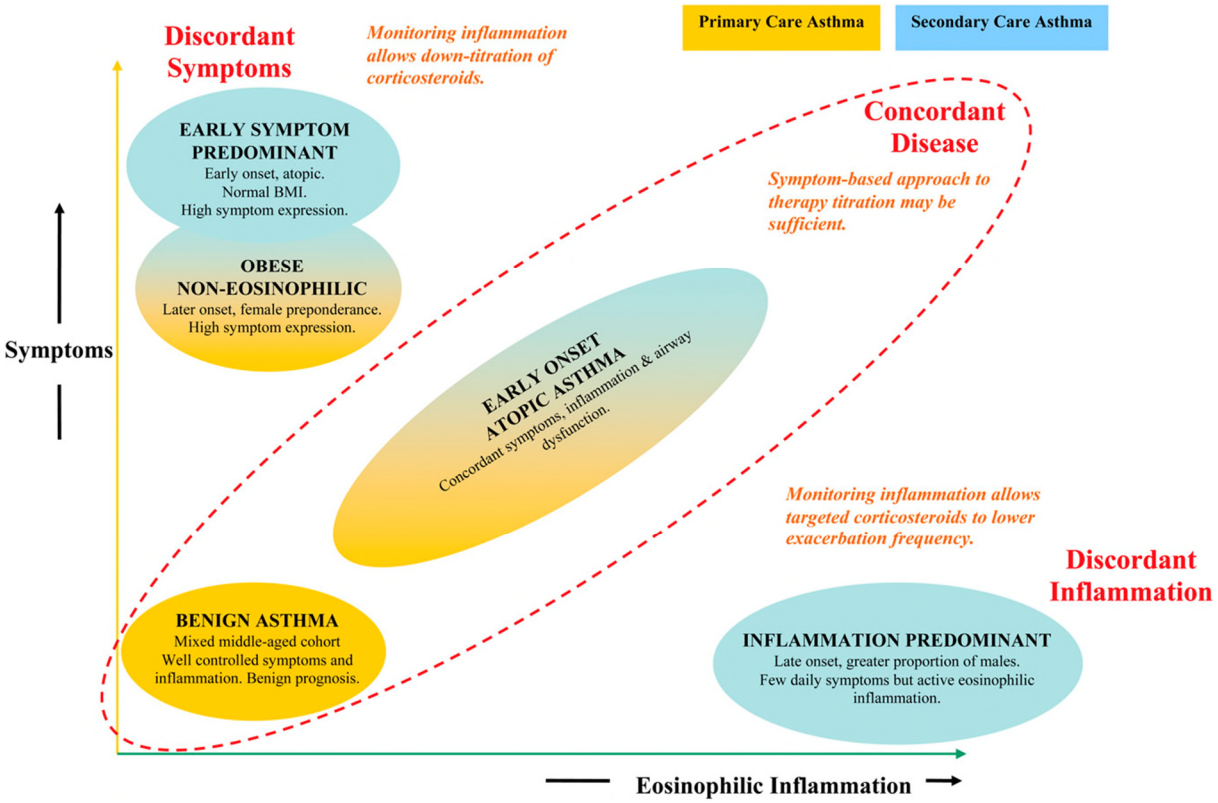


Figure 1.2: Summary of asthma phenotypes identified by cluster analysis in the primary and secondary care by the Leicester severe asthma group[5]

The definition of a true phenotype (or endotype) requires a unifying and consistent natural history, consistent clinical and physiological characteristics, an underlying pathobiology with identifiable biomarkers and genetics and a predictable response to general and specific therapies[4] (Table 1.1).

	Natural history	Clinical and physiological features	Pathobiology and biomarkers	Genetics	Response to therapy
Early-onset allergic	Early onset; mild to severe	Allergic symptoms and other diseases	Specific IgE; Th2 cytokines; thick SBM	17q12; Th2-related genes	Corticosteroid-responsive; Th2-targeted
Late-onset eosinophilic	Adult onset; often severe	Sinusitis; less allergic	Corticosteroid-refractory eosinophilia; IL-5		Responsive to antibody to IL-5 and cysteinyl leukotriene modifiers; corticosteroid-refractory
Exercise-induced	Mild; intermittent with exercise		Mast-cell activation; Th2 cytokines; cysteinyl leukotrienes		Responsive to cysteinyl leukotriene modifiers, beta agonists and antibody to IL-9
Obesity-related	Adult onset	Women are primarily affected; very symptomatic; airway hyperresponsiveness less clear	Lack of Th2 biomarkers; oxidative stress		Responsive to weight loss, antioxidants and possibly to hormonal therapy
Neutrophilic		Low FEV1; more air trapping	Sputum neutrophilia; Th17 pathways; IL-8		Possibly responsive to macrolide antibiotics

Table 1.1: One classification of asthma phenotypes based on similarity of clinical **characteristics**[4]

In essence, then, an asthma "phenotype" is characterised by common features such as clinical characteristics (age, severity, BMI, airway reversibility), triggers (allergens, aspirin, obesity), and some perception of the "degree" of inflammatory change in the airways. It might be argued that phenotypic classification remains to an extent poorly discriminatory, vague and arbitrary. It is scarcely surprising, therefore, that such classifications provide relatively little basis for understanding pathophysiology of the disease, the significance of biomarkers and the likely response to therapeutic strategies.

1.1.2 From phenotype to endotype

The concept of asthma "endotyping" is designed to move on from phenotyping towards the identification of subgroups of asthma linked by distinct functional and/or pathophysiological mechanisms[6]. Again, in view of the relative paucity of knowledge about the pathophysiology of asthma, and the uncertain relationship between the pathogenesis of bronchial hyperresponsiveness and airways obstruction and inflammation, endotyping of asthma is also not without its difficulties at present. This is well illustrated when considering the role of "atopy", as conventionally defined, in asthma: little or nothing is known about the molecular mechanisms which govern the continuous production of allergen-specific IgE in some individuals but not others, or why this production results in clinical symptoms on exposure to the relevant allergens in some individuals but not others. There is also a paucity of long term, large scale studies characterising the natural history of such mechanisms over the lifetime of an individual suffering from asthma or how these are linked with the presence of identified phenotypic traits. The process will require consideration of clinical features, pathophysiology,

biomarkers, genetics, natural history and stability. One of the several proposed or tentative asthma endotype classifications is presented in table 1.2[6].

	Natural history	**Clinical**	**Genetics**	**Pathobiology**	**Biomarkers**	**Response to treatment**
Early onset allergic	Childhood onset, severe disease, persistent & progressive	Aeroallergen sensitivity, other allergic diseases	17q12-21 Th2 pathway	Th2 cytokines, eos-less clear in severe disease	FeNo, Specific IgE, Periostin	Mild, responds to CS and Th2 blockers. Less clear in severe
Persistent eosinophilia	Adult onset, persistence and progression unknown	Often severe sinusitis, nasal polyps. Subset with AERD	LT pathway	Blood and lung eos despite CSs IL-5, cLT pathway	Sputum eos (not specific for this endotype)	Anti-Th2/IL-5 LT modifiers
ABPM	Usually adult onset persistent, but progression unknown	Increased cough/mucus central bronchiectasis	? CFTR	Blood and lung eos, mixed adaptive immunity	Fungus specific IgE and IgG	CSs, antifungals, possibly anti-IgE
Obese-Female	Very late onset persistence and progression unknown	Very symptomatic, but less airway obstruction and few severe exacerbations hormonal ties	Unknown	Inconsistent reports	Unknown	No good studies of targeted Rx, but poorly CS responsive
Neutrophilic	Unknown	Fixed airway obstruction, few other defined clinical characteristics	Unknown	Neutrophils, possibly increased innate immune activation	Unknown	Possible response to macrolide antibiotics

Table 1.2: Evolving endotypes in relation to severe asthma

There is strong evidence supporting a Th2-high phenotype in up to 50% of people with asthma of any severity, yet 50% show no evidence for this immune process. Even the percentage of early-onset allergic asthma that is truly Th2 high remains unknown.[4]

1.1.3 T cell immune responses in asthma

Prior to the 1980s it is fair to say that asthma was envisaged as a disease caused by periodic contraction of hyperreactive airways caused by the release of histamine and other mediators from IgE-sensitised mast cells on exposure to allergens. The "elephant in the room" with this concept has always been that atopy, conventionally defined as the production of a systemic IgE response to a series of arbitrary environmental proteins that we call "allergens", is neither necessary nor sufficient for the development of asthma. From the late 1980s onwards, data emerging from many studies suggested that asthma is characterised by inflammation of the airways mucosa, and more specifically by the elevated expression of Th2-type cytokines such as IL-5, IL-4 and IL-13, thought then to originate from activated, Th2-type T cells. The underlying assumption then was that these T cells are driven by immune responses to allergens (Fig. 1.4) although formal proof of this assumption has never been produced, probably because it is not in fact true. Very recently it has become clear that Th2-type cytokines can be produced in large quantities by innate immune cells within the airways mucosa such as ILC2s, and that these also strongly promote the local differentiation of Th2-type T cells and local switching of B cells to IgE synthesis. In turn these ILCs are activated by the alarmin cytokines IL-33, TSLP and IL-25, which are produced by bronchial epithelial cells in response to a wide variety of environmental stimuli such as particulate pollution, viral infections and inhaled proteins with intrinsic protease activity acting on protease-activated receptors (PARs), including some allergens such as HDM[7,8]. Thus, the emphasis has shifted away from asthmatic inflammation being driven primarily by allergen-driven responses:

(Fig. 1.6). Indeed, it could be hypothesised that the IgE production observed in some asthmatics is secondary to this alarmin-driven inflammatory process, and not the prime driver of the disease. Patients with asthma may make IgE against all sorts of proteins presented locally at the bronchial mucosa, which we normally do not classify and look for as aeroallergens, e.g. the Staph enterotoxin IgE.

At the same time, it is certainly possible that alarmins/ILC2s/Th2 T cells do not drive the airways inflammation observed in every asthmatic. For example, Th17 cells may induce neutrophilic inflammation and other possible mechanisms driving non-Th2 asthma[9-13] (Figs. 1.3 and 1.5).

Finally, airways inflammation is not the same as bronchial hyperresponsiveness (BHR), which is an inherent abnormality of bronchial smooth muscle. Airways inflammation can augment the clinical effects of BHR by reducing the internal diameter of the airways, producing a greater degree of airways obstruction for a given degree of bronchial smooth muscle contraction. Thus there will be some patients with BHR but little bronchial inflammation: these patients correspond with the "early symptom predominant" sort of patients (Fig 1.2): they have hypersensitive airways, but because they have little bronchial inflammation they have occasional, benign symptoms. Most patients have BHR with a moderate degree of airways inflammation, which responds to conventional anti-asthma therapy (the "concordant" group in Fig 1.2).

Then there are patients with BHR who also have severe airways narrowing driven by alarmins and possibly secondary remodelling of the airways, resistant to steroid reversal. With these patients, any acute exacerbation of airways narrowing by increased inflammation or mucin

production, such as by a viral URTI, is likely to result in critical airways narrowing, with repeated visits to hospital with severe asthma episodes. Anti-IL-5 biologicals reduce this inflammation somewhat by reversing some of the eosinophilic infiltration and thus increasing the internal airways diameter and thereby reducing the frequency of asthma exacerbations in treated patients. Anti-TSLP[14] (and anti-IL-33, if it ever appears), should theoretically be better than anti-IL-5 for this, resulting in a reduced incidence of exacerbations[15].

Until recently, there was no known functional linkage between BHR and airways inflammation in asthma, other than the fact that inflammation, by narrowing the lumen of the airways, greatly exacerbates the blockage arising from a given degree of bronchial smooth muscle contraction. Recent work on the calcium sensing receptor published recently in *Science*[16] reveals that this protein regulates bronchial smooth muscle intercellular calcium concentrations, causing the muscle contract more intensely in response to any contractile stimulus, which could be the main mechanism driving BHR. Further, other cationic proteins may act as agonists at this receptor, most notably eosinophilic cationic proteins and polyamines such as spermine and spermidine from neutrophils[7]. These observations provide the basis for the first ever-functional link between inflammatory cellular infiltration and direct exacerbation of BHR.

1.1.3.1 Th2 versus non-Th2 asthma

Almost since the inception of the concept that immunity can be divided into Th1 and Th2 type processes, asthma has been considered a Th2 process[4]. Although asthma has classically been considered a disease of Th2 immunity, the complex clinical heterogeneity of the disease is increasingly recognised. Th2 cells and the cytokines that they secrete are distinct features of the allergic response in atopic asthmatic patients; however, other effector T-cell populations

are also observed in the lung. Thus the molecular mechanisms that drive the underlying pathology in the allergic lung are likely to involve a more diverse panel of cytokines than the classical Th2 family. This might indicate contribution from different immune pathways in eliciting disease. Asthma is a heterogeneous disease that is driven by numerous T-cell subsets, Th2 and non-Th2, (e. g. Th17, Th9) and the interactions between genetic susceptibility and the environment determine the pathophysiology of disease[17].

1.1.3.2 Th2 associated asthma

As elucidated above, asthma has traditionally been considered a Th2 process that is linked strongly to atopy and allergy, type I hypersensitivity reactions, eosinophilic inflammation and response to corticosteroids. The data from many studies suggest that the majority of - but, clearly, not all -asthma cases fit this traditional view[5,18]. Current phenotyping approaches support the existence of an early-onset, mostly atopic and allergic asthma phenotype, and most have additionally identified a later-onset (often age 20 or later) eosinophilic phenotype. The molecular and targeted therapy data support an overall Th2 association with both of these phenotypes, such that these two clinically different yet immunologically overlapping phenotypes may fall into a broader category of Th2-associated asthma. Finally, the clinical phenotype of exercise-induced asthma (EIA) is also likely to have a Th2 component, given its eosinophil and mast cell related profile[19,20].

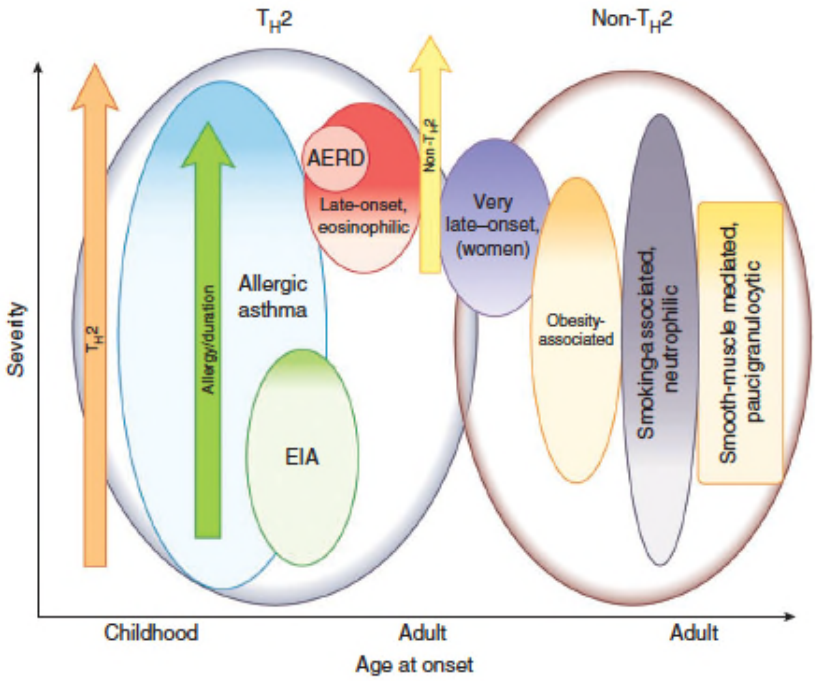


Figure 1.3: Theoretical grouping of emerging asthma phenotypes based on the distinction between Th2-high asthma and non-Th2 asthma. Th2 asthma consists of both early- and later-onset disease over a range of severities. It is likely that the majority of early-onset allergic asthma is mild but that an increasing complexity of immune processes leads to greater severity. Later-onset eosinophilic asthma without traditional allergic elements is more likely to be severe, whereas EIA is a milder form of Th2 asthma. Non-Th2 asthma includes very late–onset, obesity-associated asthma as well as smoking-related and neutrophilic asthma, and asthma in which affected individuals show little inflammation. The intensity of the colours represents the range of severity; the relative sizes of the sub-circles suggest relative proportions of affected individuals[4].

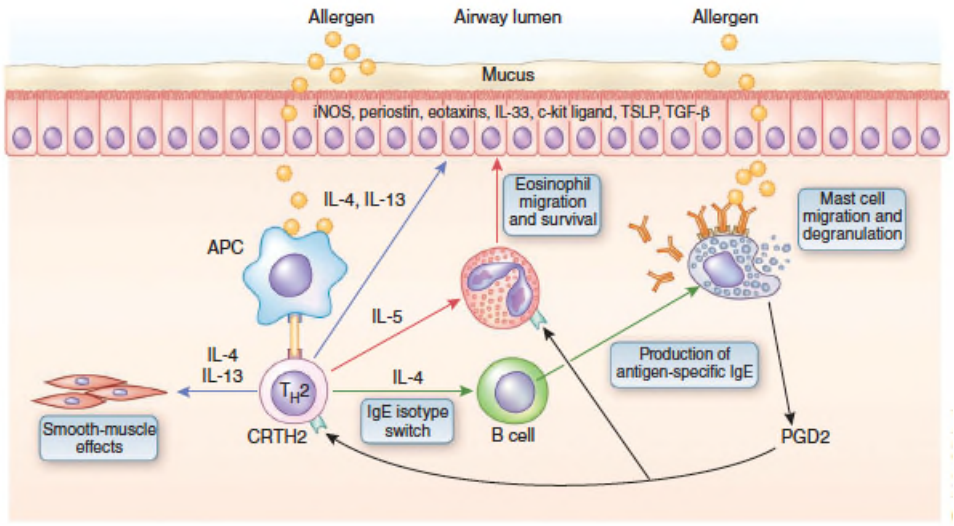


Figure 1.4: Th2 immune processes in the airways of people with asthma. The pathway begins with the development of Th2 cells and their production of the cytokines IL-4, IL-5 and IL-13. These cytokines stimulate allergic and eosinophilic inflammation as well as epithelial and smooth muscle changes that contribute to asthma pathobiology. APC, antigen-presenting cell; CRTH2, chemo-attractant receptor-homologous molecule expressed onTh2 cells; iNOS, induced nitric oxide synthase; PGD2, prostaglandin D2; TSLP, thymic stromal lymphopoietin.

1.1.3.3 Non-Th2 asthma[4]

Non-Th2 asthma is likely to represent a substantial proportion of all asthma. Compared with Th2 asthma, however, relatively little is understood about this asthma subgroup, the phenotypes underlying it or the molecular elements that control it. Non-Th2 asthma may affect 50% or more of corticosteroid-naïve individuals, who, although they meet the criteria for asthma, show less airway obstruction and hyperreactivity than people with Th2 high asthma. Many people who have mild to moderate adult-onset asthma and no history of childhood allergic features are likely to fall into this category. As these individuals also respond poorly to corticosteroid therapy, in the absence of validated Th2 biomarkers, the proportion of non-Th2 asthma in persistently symptomatic, corticosteroid-treated patients is

not clear. However, the lack of efficacy of Th2 targeted therapies in studies of non-phenotyped, corticosteroid-treated patients (even when corticosteroid treatments are tapered) and the existence of multiple other pathways by which airway hyperresponsiveness may develop strongly suggest that a subset of asthma exists with no Th2 immunity.

The major Th2 and non-Th2 asthma phenotypes and their features are listed in table 1.1. Their grouping based on clinical characteristics is illustrated in figure 1.3. The molecular and immune processes are schematically illustrated in figures 1.4 and 1.5.

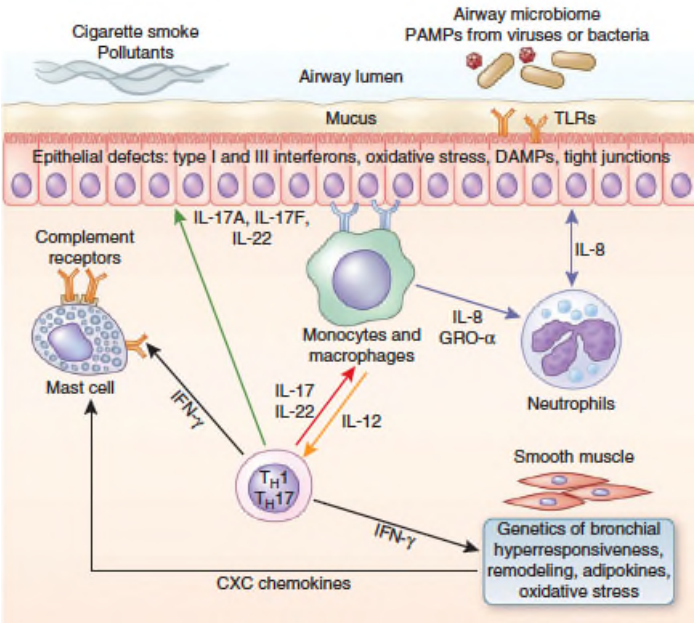


Figure 1.5: Theoretical range of factors that may be involved in the development of non-Th2 asthma. These factors include infection-related elements, Th1 and Th 17 immunity, non-Th2 associated smooth muscle changes, including genetics and oxidative stress, and the development of neutrophilic inflammation. IFN-γ: interferon-γ; GRO-α: growth-regulated oncogene-α; PAMP: pathogen-associated molecular pathway; DAMP: danger-associated molecular pathway; TLR: Toll-like receptor.

1.2 Atopic and Non-Atopic Asthma: "The Concept"

By convention, the classification of asthmatic patients as atopic or non-atopic is based on whether or not their symptoms may be precipitated or aggravated upon contact with one or more local environmental, airborne proteins recognised a being capable of inducing a specific

IgE response and acute release of histamine from mast cells and basophils sensitised by this specific IgE ("aeroallergens"), supported by the demonstration of these allergen-specific IgE antibodies as evidenced by positive skin prick and/or serological tests. Rackemann first introduced the terms "extrinsic" and "intrinsic" asthma in 1947[21]. He observed in a study of a limited number of patients a form of the disease in which extrinsic factors were triggering the symptoms and another in which no specific triggering factor could be identified. He called the former "extrinsic asthma" and the latter "intrinsic asthma". According to Rackemann, "extrinsic" asthma was easier to diagnose, associated with vasomotor rhinitis, allergy and of onset before the age of 30. "Intrinsic" asthma was more difficult to diagnose and associated with polypoid sinusitis. More recently, and not necessarily in line with Rackemann's view of external "triggers", the differentiation between the two entities has been focussed on the presence or absence of positive skin prick and/or serological tests to common aeroallergens rather than purely on the basis of a clinical history of symptoms on allergen exposure. Nowadays, these terms have become equated with the presence or absence of co-existing atopy, defined as the propensity of some individuals to mount specific IgE responses to certain antigens ("allergens") encountered at mucosal surfaces as detected by skin prick or *in vitro* tests. Although in allergic ("extrinsic") asthmatics exposure to allergens may cause asthma exacerbation, other environmental influences (such as viral infections) more commonly exacerbate asthma regardless of atopic status. A series of studies designed to analyse the immunopathological differences between atopic and non-atopic asthma resulted in the conclusion that both kinds of asthma share more similarities than differences[22,23]. Nieves et al. conducted a relatively large population study in 751 asthmatics to understand the discriminating characteristics between the two groups and concluded that despite the immunopathological similarities, atopic and non-atopic asthma reflect distinct clinical groups[24]. Age, age of onset of asthma, and female/male ratio were higher in non-atopic than

in atopic asthmatics. Conversely, atopic asthma typically commenced at an earlier age with a higher male/female ratio and was more associated with rhinitis and smoking. In the above survey, about a quarter of patients were non-atopic, whereas an earlier Swiss SAPALDIA survey concluded that a third of total asthmatics were non-allergic (intrinsic)[25,26]. An even higher prevalence of non-atopic asthma has been reported by some later studies: for example, the prevalence of non-atopic, severe asthma was estimated at 50% of the total in the ENFUMOSA cross sectional study[27] and 17–34% in the SARP study[18]. Early epidemiological studies suggested that non-atopic asthmatics form an identifiable clinical group (more likely female, late onset, severe disease)[28]. These clinical distinctions between atopic and non-atopic asthma led to speculation about their causation being fundamentally different, in particular regarding the involvement of IgE-mediated responses[29].

Ever since the first description of intrinsic asthma, because of the all-pervading concept that asthma must be driven by "allergic inflammation" in the airways, there has been debate about its pathogenesis[26]. The fact that many asthma exacerbations, and consequently many first presentations of asthma are brought about by upper respiratory viral infections led to the suggestion that non-atopic asthma may represent a form of autoimmunity triggered by a viral infection such as influenza. Certainly, non-atopic asthmatics frequently demonstrate positive autologous serum skin tests, evidence of circulating histamine releasing factors and positive anti-nuclear auto-antibodies[30]. It has also been postulated that "non-atopic" asthmatics manufacture IgE against as yet undetected or unidentified "allergens" and may therefore benefit from allergen avoidance[31,32] (Figure 1.6). For example, the airways epithelium of asthmatics may sometimes be colonised by microbes such as *Staphylococcus*, which produce superantigens. Superantigens produced locally in the airways may lead to class switching of local B-cells, resulting in polyclonal IgE production in the airways and also the production of

specific IgE against the these superantigens[33,34]. This may also promote production of IgE against other "allergens". Superantigens can also stimulate clonal expansion of T-cells, resulting in clonal activation of Th2 cells and CD8+ cells and suppression of regulatory T cells[35,36].

While exposure of some asthmatic patients to some aeroallergens to which they are clinically sensitised may be a significant acute exacerbating factor for their symptoms (for example, seasonal or "pollen" asthma), the actual role of IgE-mediated reactions in asthma pathogenesis *per se* remains obscure. Arguably, we are moving away from thinking of asthma as being "caused" by "allergic inflammation" dependent on the presence of IgE directed against an arbitrary range of aeroallergens. It is becoming more and more clear that what we are accustomed to call "allergic inflammation" is unlikely driven by allergen-specific responses at all, and indeed that at least some IgE production may arise as a consequence of alarmin-driven Th2-type inflammation with B cell switching to IgE within the bronchial mucosa. It is hardly surprising, therefore, that the profiles of inflammatory cellular infiltration of the airways in "extrinsic" and "intrinsic" asthma are essentially the same (see next section). Extrapolating from the hypothesis that switching of local B cells to IgE producing plasma cells is a consequence, rather than a cause of the airways inflammation in asthma, it is conceivable that at least some of the resulting local mucosal IgE production is not directed at what are conventionally called "aeroallergens", but other proteins which have access to the airways through inhalation or because, for example, they are produced by commensal microorganisms such as *Staphylococcus* referred to above. In conclusion, the distinction between "allergic" and "non-allergic" inflammation may not be as distinct as Figure 1.7 suggests, and potentially all of these mechanisms are part of epithelial alarmin-driven inflammation of the airways. Certainly, previous studies on this topic have revealed

remarkable similarities in the cellular and molecular immunopathology of atopic and non-atopic asthma and the possibility of a role for IgE antibody in both clinical phenotypes of disease rather than confirming distinct pathogenetic mechanisms[22,23,37-40].

1.3 Atopic and non-atopic asthma, similarities and differences:

The pathogenic mechanisms of non-atopic asthma in comparison to its atopic counterpart have always been a subject of debate. Although clinically are two distinct entities, more similarities than differences have been observed between the two in terms of immunopathogenesis, underlying IgE mechanisms, and so on. More information has become available in recent years comparing the ultrastructure of the epithelium in these two types of asthma. A comparison of airway epithelium/immunopathology in atopic and non-atopic asthma is presented in the figures (Figures 1.6 & 1.7) and tables (Tables 1.3, 1.4 & 1.5) in the following pages, from the available information in the literature[22,37-53]. Similarities outnumber the differences, until we unravel the mystery surrounding these two important phenotypes of asthma in more detail.

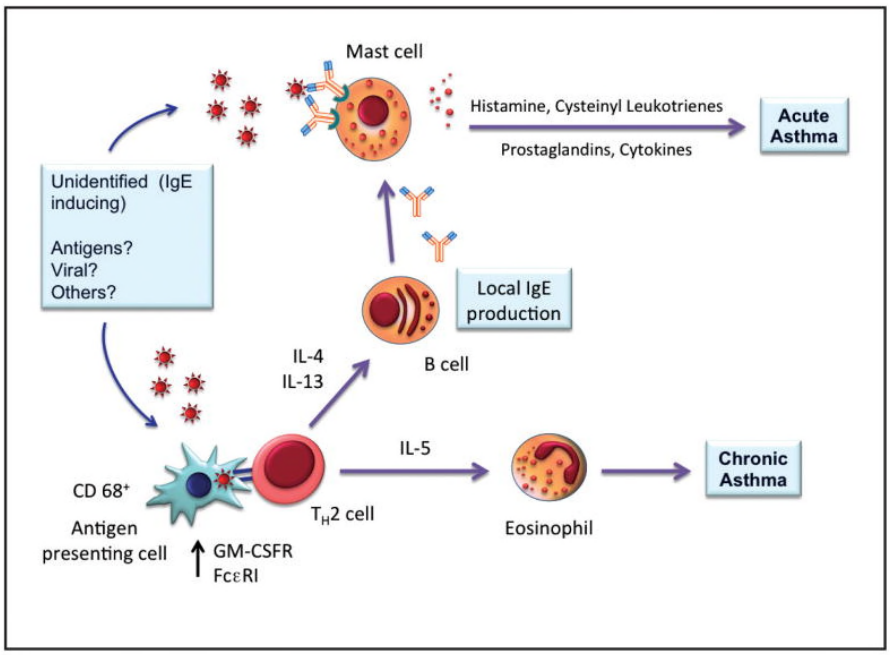


Figure 1.6: One theoretical, postulated mechanism of non-atopic asthma. In the absence of atopy, unknown antigens could presumably trigger local IgE production. Such antigens, as in atopic asthma, lead to the release of pharmacological inflammatory mediators that cause rapid onset bronchospasm and chronic asthma[54]

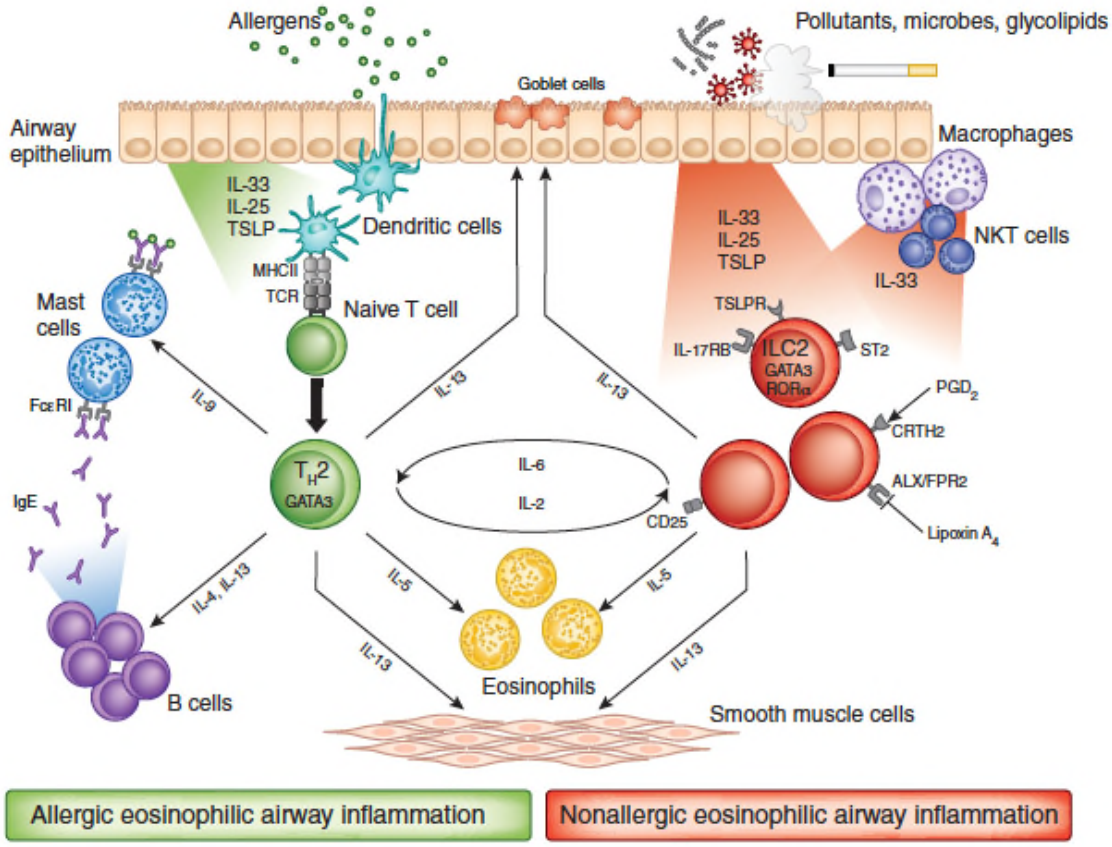


Fig 1.7: Two different pathways leading to airway inflammation in asthma[55]. In allergic asthma, dendritic cells present allergens to CD4+ T-cells, inducing T-helper (Th2) cells, which produce interleukin IL-4, IL-5 and IL-13, and leading to IgE switching in B-cells, airway eosinophilia and mucous hypersecretion. In non-allergic eosinophilic asthma, air pollutants, microbes and glycolipids induce the release of epithelium-derived cytokines, including IL-33, IL-25 and thymic stromal lymphopoietin (TSLP), which activate innate lymphoid cells (ILCs) in an antigen-independent manner via their respective receptors: IL-17 receptor B (IL-17RB), ST2 and TSLP receptor (TSLPR). Activated ILC2s produce high amounts of IL-5 and IL-13, leading to eosinophilia, mucus hypersecretion and airway hyperreactivity. CRTH2: chemo-attractant receptor homologous molecule expressed on Th2 cells; ALX/FPR2: receptor for lipoxin A4; FcεRI: high-affinity receptor for IgE; GATA3: GATA-binding protein 3; PG: prostaglandin; ROR: retinoic acid receptor-related orphan receptor; NK: natural killer; MHC: major histocompatibility complex; TCR: T-cell receptor.

Cell types or epithelial components	Atopic asthma	Non-atopic asthma
Ciliated columnar	Damage +++	Damage +
Desmosomes	Breakdown ++	Breakdown +
Goblet cells	Hyperplasia+	Hyperplasia -
Basal cells	Damage +/-	Damage +/-
Basement membrane	Thickening++	Thickening+
Eosinophils	Infiltration+++	Infiltration+++
Neutrophils	Infiltration+	Infiltration++
Mast cells	Infiltration++	Infiltration+
Lymphocytes	Infiltration+++	Infiltration++
Macrophages	Infiltration+	Infiltration++

Table 1.3: Comparison of bronchial epithelial components in atopic and non-atopic asthma[56]

Cytokines and receptors		Atopic asthma	Non-atopic asthma
Th1	γ IFN (sputum)	++	++
	IL-2 (BAL fluid)	+	++
	IL-2R+	+	+
Th2	IL-4	++ +/-	++
	IL-4α Receptor	++	++
	IL-5	++ +/-	++
	IL-5α Receptor	++	++
	IL-10 (Sputum)	++	+
	IL-13	++	++
	GMCSF	++	++
Th1&Th2	IL3	++	++
Macrophage	GMCSFR-α	+	+++

Table 1.4: Cytokine and cytokine receptor expression in the respiratory epithelium and sub mucosa[56]

IgE related expression	Atopic asthma	Non-atopic asthma
CD20+ B cells	++	++
FcεRI+ cells	++	++
ε-GLT	+++	+++
Iε-CμCT	++	++
Iε-CγCT	++	++
ε-mRNA	+++	+++
AID-mRNA	++	++

Table 1.5: IgE and related molecules in the respiratory mucosa[56]

1.4 IgE and its role in allergy

IgE, the fifth and least abundant class of immunoglobulins, is thought to have evolved in mammals as a first line defence mechanism against parasites, particularly helminths and protozoa. Today it is perhaps better known as a mediator of type 1 hypersensitivity or allergic reactions ranging from allergic rhinitis and asthma to life-threatening anaphylaxis. IgE is the antibody potentially capable of evincing clinical sensitivity to allergens. It shares the same basic molecular architecture as antibodies of other classes, with two identical heavy chains and two identical light chains (Fig: 1.8). IgE has 4 heavy chain domains (Cε1-Cε4) and differs from other antibody isotypes by being located predominantly in tissues, bound to mast cells and basophils through its high-affinity receptors. In this cell-bound state IgE can persist for extended periods of time. Most of the IgE is retained in tissues, and free serum IgE concentrations are the lowest of all immunoglobulin classes. IgE does not activate complement and exerts functions principally through its receptors. The high-affinity receptor "FcεRI" is expressed on basophils and mast cells as a αβγ2 tetramer and on Langerhans cells, myeloid dendritic cells (DCs), plasmacytoid DCs, monocytes and eosinophils as a αγ2 trimer. The low-affinity receptor FcεRII (CD23) is a C-type lectin and of two types- CD23a and CD23b. CD23a can be identified on activated B cells, and CD23b on a wide variety of cells such as B cells, macrophages, eosinophils, natural killer cells, T cells, follicular dendritic cells, airway epithelial cells and smooth muscle cells [57].

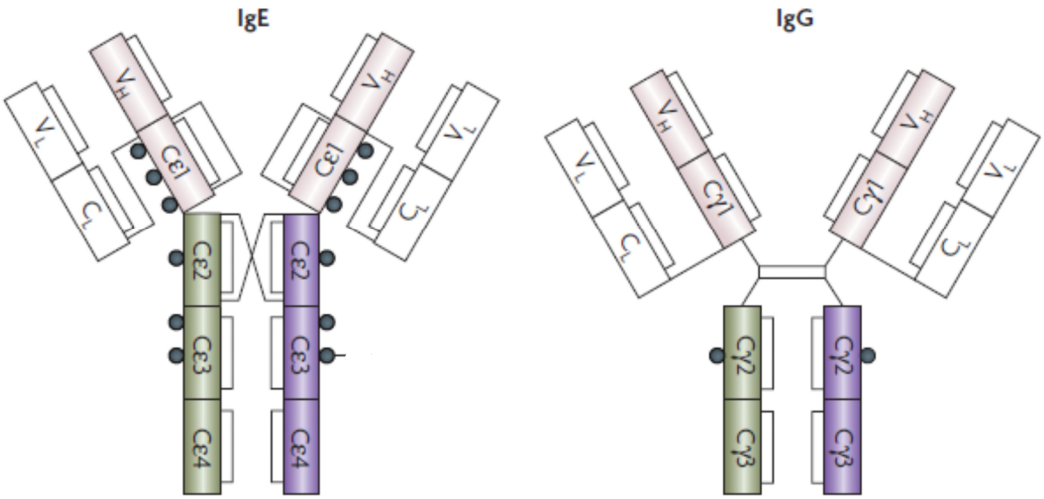


Fig: 1.8 Basic molecular structure of IgE (in comparison with IgG)[58]

The discovery and characterization of the antibody class now called IgE[59] , culminating in the independent descriptions of this class of antibodies by the Ishizakas[60] and Johansson and Bennich[61], arguably represents the most crucial advance in our understanding of the immunological basis of allergic disorders. Production of antigen-specific IgE requires that such antigens are taken up by dendritic cells, B cells or other antigen-presenting cells which, in the presence of interleukin-4 (IL-4) or IL-13 provided early in the process by one or more cell types, present the processed antigens to cognate naive T cells that then acquire a T helper type 2 (Th 2) cell phenotype[62]. Th 2 cells both engage cognate B cells through B cell major histocompatibility complex (MHC) class II and co-stimulatory molecules and secrete IL-4 and IL-13, inducing B cells to undergo class-switch recombination (CSR), resulting in the variable, diverse, and joining (VDJ) segments initially linked to another constant (C) region in the immunoglobulin heavy chain locus (for example, Cμ or Cγ) to instead being linked to the Cε region. CSR may also be induced by IL-4 and/or IL-13 derived from cells other than Th 2 cells, which may include mast cells and basophils[62-64].

Antigen sensitization was previously thought to occur primarily in lymphoid germinal centres, but IgE-producing B cells that undergo clonal selection and affinity maturation may also be generated in the mucosal associated lymphoid tissue including that of the respiratory mucosa[65] . CSR resulting in production of IgE (in addition to IgA) also can occur in the gastrointestinal tract, and patients with food allergy have higher concentrations of IgE in the gastrointestinal tract than healthy individuals[66]. Such evidence supports the conclusion that IgE can be produced locally by B cells in the gut- or airway-associated lymphoid tissue, as well as in the lymph nodes, of individuals with food allergy, seasonal or perennial allergic rhinitis[67] or atopic or non-atopic asthma.

These observations suggest that much of the IgE responsible for 'organ-specific' allergic disorders may be produced locally in the affected anatomical sites, which also may be a survival niche for long-lived IgE-antibody–secreting plasma cells, and that IgE measured in the peripheral circulation may be primarily antibody that has escaped from the site of disease. Indeed, concentrations of IgE in the peripheral blood are typically much lower than those of any other immunoglobulin isotype[58]. These findings also suggest that locally produced IgE may be pathogenic in at least some cases of so-called non-atopic asthma under circumstances in which it may be difficult to detect crucial IgE antibodies systemically, particularly in individuals in which no triggering antigen has been identified. [65]

The sites of IgE production in human subjects and the nature and characteristics of IgE-producing cells are of great interest. In this context, it has been shown that allergen-specific IgE levels can be boosted by contact with allergens via the respiratory mucosa of the nose. Also, it has been proposed that allergy effector organs (e.g., the nasal mucosa and the lung) may be important sites of IgE production in allergic patients. IgE-producing cells have also

been found in the blood, but their numbers are extremely low. Transfer of specific sensitization during bone marrow transplantation indicates the presence of IgE-producing B memory cells or plasma cells also in the bone marrow.

The half-life of free serum IgE is rather short in comparison to other immunoglobulin classes -12 h in mice[68] and 2–3 days in humans[69,70]. Although the survival of IgE antibody can be markedly increased by binding to its high affinity receptor, FcεRI, or its low affinity receptor, CD23[71], it has to be produced continuously in order for serum concentrations to be maintained. The investigation of IgE production and metabolism in humans is hampered by the small numbers of IgE-producing cells in blood, nasal mucosa or tonsils. Therefore, our knowledge of the location and precise mechanisms of IgE production and of the metabolism of IgE-producing cells is mostly based on extrapolation of data obtained in mouse models and *in vitro* systems using peripheral blood mononuclear cells (PBMCs) or tonsil-derived B cells. Based on our current knowledge, the potential locations of IgE production are blood, nasal mucosa, lungs, adenoid, tonsils, spleen and bone marrow. After leaving the bone marrow, mature IgM- and IgD bearing B cells with specificity for allergens become activated upon recognition of their cognate antigen. Subsequently, they interact with T cells, which have previously been activated by allergen presentation through professional antigen-presenting cells. The cytokines interleukin (IL)-4 and IL-13 produced by activated T helper 2 (Th2) cells promote the class switch to IgE[62]. Whether the switch to IgE occurs directly or via other switch stations is a matter of debate. In humans, switching to IgE can occur apparently either directly or via IgG of all four classes[72-74]. Switching via Sγ was demonstrated in a human cell line[75] as well as in nasal mucosal samples from allergic patients[74]. In a group of children from a birth cohort who acquired allergy during their first 7 years of life, indices of both direct and sequential class switching were observed[76].

Whether IgE memory in human allergic patients is due to the existence IgE memory cells or is sustained by long-lived IgE plasma cells and a pool of IgG memory B cells with specificity for allergens that switch to IgE production upon allergen contact is currently under debate[57,77-81]. So far, only a few sites in the human body have been thoroughly investigated directly for the presence of cells involved in allergen-specific IgE memory. Although IgE antibodies and their role in allergic disease were first described in 1967[82], the source that continuously replenishes serum IgE levels is still far from clear. It appears unlikely that a small number of cells present in the peripheral blood could constantly produce as much allergen-specific IgE as is needed to arm mast cells and basophils for their effector function. In this context, it is likely that approximately 0.2 % of the serum IgE is newly produced by blood-derived plasma cells[83]. The current observations indicate that there are few IgE-producing cells in the blood, but that the majority of IgE-producing cells in human allergic patients reside elsewhere. In previously sensitized allergic patients, respiratory contact with relevant allergens is followed by a substantial systemic elevation of specific IgE antibodies[84-87]. It has been proposed that this allergen-contact induced elevation of IgE antibody production may arise locally in the airways mucosa. The presence of allergen-specific IgE in nasal secretions of allergic patients was recognised as early as 1979[88]. Recent clinical studies have revealed that a substantial number of patients with rhinitis without detectable, serum allergen-specific IgE and with negative skin prick tests mounted a positive response to specific allergens in nasal provocation tests and also had specific IgE antibodies in nasal secretions[89,90]. Nasal mucosal biopsies from symptomatic allergic rhinitis patients but not from non-allergic controls contained increased numbers of IgE-positive B cells and plasma cells and allergen-specific plasma cells[67]. To investigate whether these IgE-producing cells derived from more distant lymphatic tissue or whether they were resident to the nasal mucosa and performed class

switch to IgE locally, biopsies of nasal mucosa from allergic patients were challenged *ex vivo* with allergen[73,74,91-93]. Interestingly, not only local synthesis of ε germline transcripts[92,93], but also ε circle transcripts as signs of mature IgE synthesis[73,74] could be detected in the biopsies upon allergen challenge. Upon inhalation of airborne allergens, not only the nasal mucosa but also other lymphatic tissue of the airways, such as the adenoids and the tonsils as part of the Waldeyer's lymphatic ring, are potential sites of allergen encounter. In this context, tissue homogenates of adenoids of atopic children showed higher levels of total and allergen-specific IgE compared to non-atopic children[94]. Using immunohistochemical staining, IgE+ cells were identified mainly in the extrafollicular area of the adenoids of atopic subjects, whereas adenoids of healthy controls contained only very few IgE + cells[95]. Those IgE + cells were identified mainly as plasma cells and macrophages using double staining for IgE and the plasma cell marker CD138 or the macrophage marker CD68, respectively[96]. Furthermore, tonsil-derived PBMCs also produced IgE when cultured in vitro and stimulated with pokeweed mitogen[97,98]. The presence of allergen-specific IgE in the sputum of house dust mite-sensitised asthmatic patients indicates that local IgE production may also occur in the airways of patients suffering from allergic asthma[99]. Analysis of IgE transcripts of a bronchial biopsy of a polysensitised asthmatic patient indicated that IgE-synthesising B cells may also be found in the airways of humans[100]. However, class switch recombination to IgE has been observed in bronchial biopsies of both atopic and non-atopic asthmatic patients but not in atopic control subjects not suffering from asthma[53,101]. Thus, local IgE synthesis in the lung seems to be one characteristic of asthma regardless of the conventional atopic status of the patient potentially due to the fact that the high alarmin/high Th2 environment typical of asthmatic inflammation may be conducive to B cell switching to IgE in all asthmatics, regardless of their conventional atopic status. IgE-producing cells were detected in the spleen of aerosol-challenged mice that had previously sensitised to ovalbumin[102]. Whether this also

applies for human allergic patients is so far not clear, as data on the presence of IgE producing cells in human spleen are rare. One report analysing the spleen of an atopic patient sensitised to house dust mite who died from an asthma attack, showed that IgE was also produced in the spleen[103]. However, the nature of the IgE-producing cells and the specificity of the IgE antibodies produced were not reported.

Some case reports and small studies investigated the effect of transferring bone marrow from an atopic donor into a non-atopic recipient[104-109]. The results are ambiguous. In some case reports, non-atopic recipients receiving bone marrow from atopic donors developed sensitisation profiles similar to those of their donors[104-107]. However, it has also been shown that not all recipients acquire the allergic profile of their donors[108] and that some of the patients also acquire *de novo* allergic sensitisations in addition to those transferred from the donor[109]. Thus, IgE memory seems to be transferable; however, to which extent and which cells – long-lived plasma cells or memory B cells – are responsible remain to be addressed.

1.5 The concept of allergen sensitisation, IgE production and facilitated antigen presentation[110] (Figure 1.9)

Initial allergen sensitisation results in antigen-specific IgE production (left half of the figure 1.9). In individuals not yet exposed to a new environmental allergen, the only IgE present (blue in the figure 1.9) does not have specificity for the new antigen(s). Such IgE can be bound to the αβγγ form of FcεRI on mast cells or to the αγγ form of FcεRI on the surface of macrophages, monocytes or dendritic cells or to CD23 on airway epithelial cells or other cells. The new antigens (orange circles in the figure 1.9) are captured by dendritic cells or macrophages in the airway lumen or in the epithelium of the airway mucosa or gain access to

sub-mucosal dendritic cells through disrupted epithelium or, for some antigens with intrinsic protease activity, by disrupting epithelial cell tight junctions. Antigen-activated dendritic cells mature and migrate to regional lymph nodes or to sites in the local mucosa, where they present processed antigen epitopes to cognate T cells; in the presence of IL-4 or IL-13, which may be derived from a variety of potential cellular sources, this induces such T cells to become differentiated and activated Th 2 cells. The key molecular switch for Th2 development is interaction of the T cell accessory molecule OX40 with its ligand OX40L expressed by dendritic cells primed by TSLP[111]. Thus again, it is likely this epithelial alarmin, which creates the environment in the respiratory mucosa for Th2 T cell differentiation. The OX40/OX40 ligand interaction activates NFAT (Nuclear Factor of Activated T-cells) which in turn triggers IL-4 production by the T cell, which by acting on its own IL-4 receptors in turn activates the key molecular T cell switches for Th2 development: STAT6 and GATA-3[112]. The Th2 cytokines IL-4 and IL-13 also activate immunoglobulin heavy chain gene CSR for antigen-specific IgE production, designated antigen-specific IgE, in B cells. The antigen-specific IgE response is amplified by FAP and other mechanisms (right half on the figure 1.9). Antigen-specific IgE can bind to multiple cell types through IgE receptors. Antigen-induced aggregation of IgE bound to FcεRI stimulates mast cell degranulation and the release of mediators such as histamine, PGD2 and TNF, which promote recruitment of Th2 cells, the migration, maturation and activation of dendritic cells and antigen presentation. IgE and antigen-IgE complexes can cross the epithelium by transcytosis mediated by CD23 on airway epithelial cells (① on Fig 1.9), allowing them to bind to and activate FcεRI on mast cells and dendritic cells. This process contributes to the perpetuation of allergic inflammation and, potentially, through promotion of IL-4 and/or IL-13 secretion by mast cells (② on Fig 1.9) and effects of activated mast cells on dendritic cells (③ on Fig 1.9), to additional local IgE CSR and IgE production in B cells, either to additional epitopes of the original antigen or to

new antigens bound by dendritic cells (square blue symbols in figure 9). As discussed previously, we do not yet understand whether the inflammation seen in the airways of asthmatics is truly "allergic inflammation" or alarmin-driven inflammation with the production of IgE playing a minor role or possibly epiphenomenal. Antigen presentation mediated by binding of antigen-IgE complexes to CD23 on B cells, followed by antigen presentation by these B cells to cognate T cells, is called Facilitated Antigen Presentation (FAP) (④ on Fig 1.9), a process that can result in epitope spreading, with production of IgE recognising new epitopes of the original antigen or to epitopes of new antigens, and the subsequent precipitation of allergic symptoms. FcεRI αγγ trimers on other antigen-presenting cells (e.g. dendritic cells, monocytes and macrophages) permit these cells to bind and internalise IgE bound to complex antigens; epitopes derived from such antigens, including those comprising epitopes for which there is not yet a specific IgE response, are then presented to cognate T cells, which, in the presence of IL-4 and/or IL-13, can become Th 2 cells that in turn promote the production of IgE against these new epitopes by B cells (⑤ on Figure 1.9).

Initial sensitisation leading to antigen specific IgE production	Amplification of antigen-specific IgE response by FAP and other mechanisms

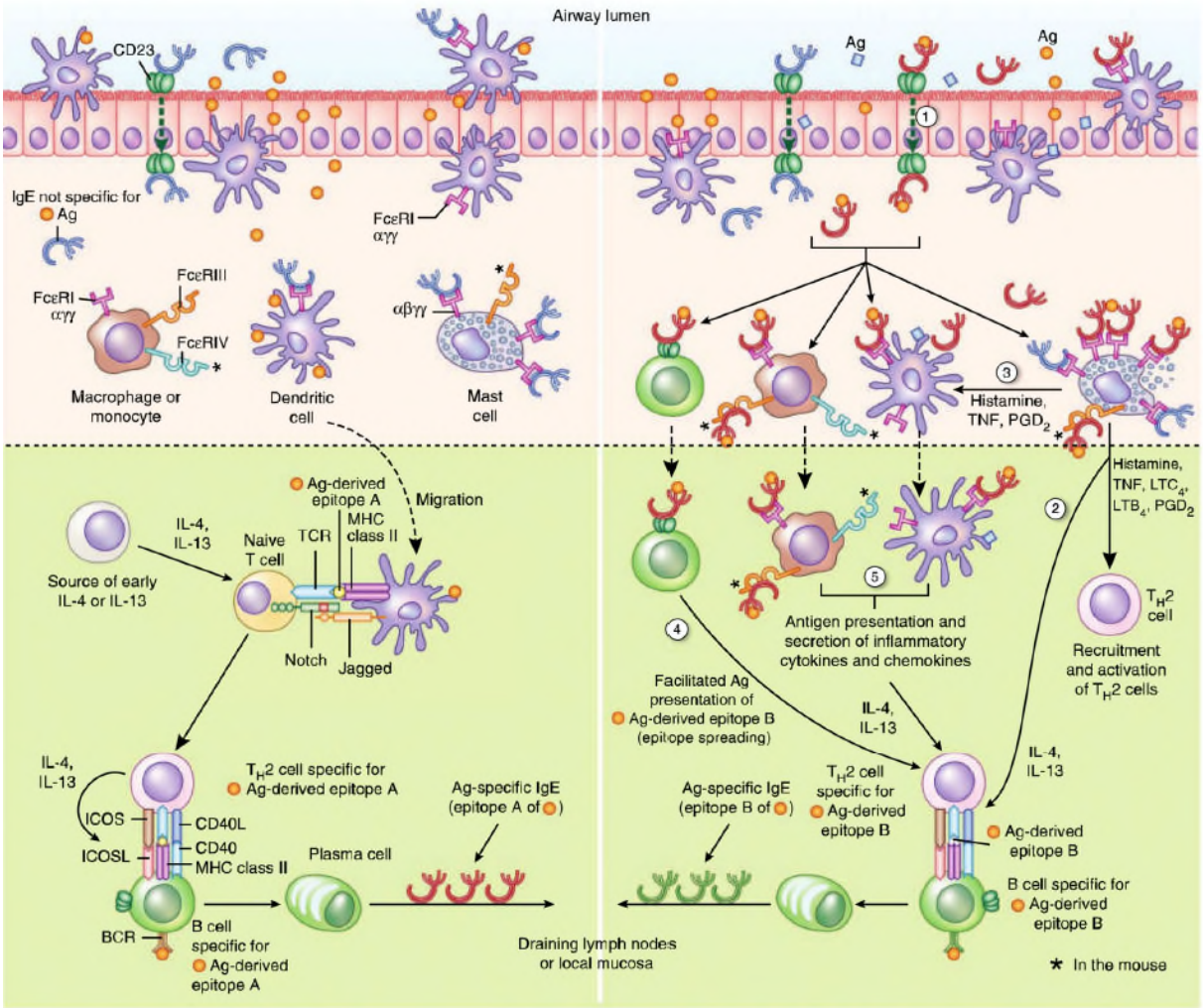


Figure1.9[110]: Allergen sensitisation and IgE production

(Ag: environmental allergen/antigen, ICOS: Inducible T Cell Co-Stimulator, ICOSL: ICOS ligand, BCR: B Cell Receptor)

1.6 IgE in atopic asthma

Allergic asthma and allergic rhinitis/conjunctivitis are characterised by a Th2-dominated immune response associated with detectable circulating IgE specific for inhaled aeroallergens. Symptoms of atopic asthma are intermittent attacks of breathlessness, wheezing and cough after exposure to inhaled allergens or other potential triggers such as viral infection. These appear to be caused by chronic inflammation and remodelling of the conducting airways on a background of bronchial hyperresponsiveness. Atopic rhinitis or conjunctivitis is an inflammation of the nasal passages or conjunctiva, usually associated with swelling, watery nasal discharge, and itching of the nose and eyes. The association between allergen specific serum IgE levels and asthma was established through epidemiologic studies. Burrows et al found a close correlation between serum total IgE concentrations, skin test reactivity and asthma. Antigens involved are mostly indoor aeroallergens derived from, for example, house dust mite, animal dander, cockroach, and moulds[113]. But more clarity is needed here to distinguish cause and effect: does IgE "cause" asthma or is elevated IgE production a bystander product of alarmin-driven mucosal inflammation in asthma, or both? This question is further complicated by the fact that the amount of allergen-specific IgE detectable by SPT/*in vitro* testing bears no relationship to the severity of the reaction, if any, to allergen exposure. Might allergen-specific IgE play a role in asthma even if it does not cause any immediate clinical symptoms? It is not clear whether IgE is capable of causing or exacerbating asthma without causing acute symptoms, possibly by activation of effector cells such as eosinophils or facilitated antigen capture and presentation by B-cells or APCs. Furthermore, asthma correlates best in epidemiological studies with total circulating IgE, not allergen specific IgE. Does this mean that IgE causes asthma regardless of its specificity, or is it that individuals who are more prone to develop alarmin-induced inflammation of their airways are more likely to manufacture IgE? Are there common genetic and/or environmental

influences which lead to both asthma and excessive IgE in the system, either due to increased production and/or reduced breakdown or removal from the circulation?

1.7 IgE in "non-atopic" asthma

Asthmatic patients who do not respond to common aeroallergens on skin prick testing or have negative serological tests to the same are described as non-atopic. About a third of adult asthmatic patients are non-atopic by this definition. Non-atopic asthmatic patients usually have more severe and difficult-to-control disease[28]. They also have increased total serum IgE concentrations compared with those seen in healthy control subjects, although experimental "asthma" can be induced in animals in the absence of B cells or IgE, an observation compatible with the hypothesis that IgE is not indispensible for the development of asthma[114-117].

Previous studies comparing the cellular and molecular immunopathology of atopic and non-atopic asthma have revealed remarkable similarities, consistent with the hypothesis that, if IgE does play some role in the pathogenesis of asthmatic airways inflammation, this is regardless of the presence or absence of conventional "atopy". Thus:

(i) The bronchial mucosal inflammatory cellular infiltrate is mostly identical except for an excess of tissue macrophages in non-atopic patients[118]. Furthermore, there is equivalent, elevated expression of the cytokines IL-4 and IL-13, which are the only human cytokines capable of isotype switching B cells so that they make IgE antibodies[22,119,120]. There is also equivalent, elevated expression of a wide range of cytokines, chemokines and their receptors implicated in asthma pathogenesis[121-124].

(ii) It has long been known from epidemiological studies that high total serum IgE concentration is a risk factor for airways obstruction[113]. A more recent study showed that in a large unselected cohort of non-atopic patients defined by negative skin prick tests, asthma was fivefold more prevalent and airways obstruction more severe in those subjects with serum IgE concentrations in excess of the "normal" upper limit of 150 IU/ml[125]. These studies make no reference to the specificity of the IgE, and indeed suggest that inappropriate IgE production is a risk factor for asthma independently of specific IgE responses to allergens.

(iii) More compelling evidence for local IgE synthesis has come from the demonstration of elevated expression of IgE ε heavy-chain germ line and mature gene transcripts in the bronchial mucosa of non-atopic as well as atopic asthmatics compared with controls in the absence of elevated numbers of B cells[126]. This observation has been further ratified by the demonstration of IgE switch circle transcripts in the bronchial mucosa of both atopic and non-atopic asthmatics but not controls[65], confirming that the mucosal environment in asthmatics is conducive to heavy-chain switching to IgE. This has led to the hypothesis that the respiratory mucosa is a major site of IgE synthesis[127], and that most of this IgE is sequestered locally bound to high and low affinity IgE receptors, with circulating IgE representing no more than the "spill over" from this process.

(iv) Cells in the bronchial mucosa of non-atopic as well as atopic asthmatics show elevated expression of the high-affinity IgE receptor FcεRI[39]. IgE in concentrations above 1 μg/ml (an order of magnitude greater than that in normal serum) up-regulate receptor expression by preventing proteolytic cleavage at the cell surface[128]. Consistent with the

effector activity of this IgE, mast cells are seen to be degranulated in the bronchial mucosa of both atopic and non-atopic asthma patients[129].

Given these findings it seems perfectly conceivable that IgE elaborated in the respiratory mucosa might not escape into the peripheral circulation in sufficient quantities to be detectable by skin prick or *in vitro* tests. Furthermore, it is perfectly possible that not all IgE responses relevant to asthma pathogenesis are directed against allergens. For example, there is evidence that IgE against respiratory tract viruses[130], Staphylococcal superantigens (which has been shown to cause local oligoclonal activation of mucosal IgE+ B cells[34,131]) and even autoantigens[132] may play a role in asthma pathogenesis. Finally, certain species of so-called "highly cytokinergic" IgE can affect the function of mast cells by mechanisms other than surface cross-linking[133,134]. All of these observations provide strong support for the hypothesis that IgE plays a role in the pathogenesis of asthma, regardless of the atopic status of the patient as defined by positive or negative skin prick tests. A corollary of this hypothesis is that anti-IgE strategies will be effective in treating patients with non-atopic asthma.

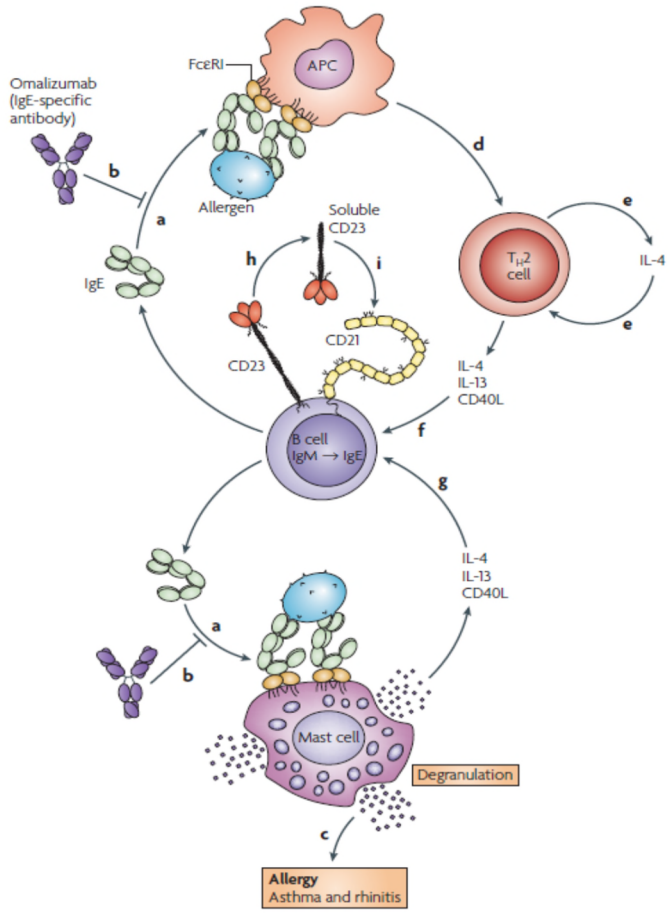


Fig:1.10[58] IgE in the pathogenesis of asthma and allergy and the role of anti-IgE

*Notes: IgE is synthesised and secreted by B cells/plasma cells that have undergone heavy-chain class switching from IgM or IgG to IgE. The IgE binds to FcεRI on mast cells and antigen-presenting cells (APCs) (**a**) and sensitizes these cells to allergens. Omalizumab inhibits the binding of IgE to FcεRI on mast cells and APCs (**b**). Allergen binding to IgE triggers mast-cell degranulation to cause an allergic response (**c**) Allergen binding to the APC leads to the presentation of allergenic peptides to T helper 2 (Th2) cells (**d**) The allergen-activated Th2 cells secrete interleukin-4 (IL-4) (**e**) to maintain the Th2-cell lineage and recruit more Th cells into this lineage (**e**) The Th2 cells also secrete IL-13 and express CD40 ligand (CD40L), which together with IL-4 stimulates heavy-chain class switching to IgE (**f**). The allergen-activated mast cells contribute to the production of IL-4 and IL-13 (and express CD40L), which may also stimulate heavy-chain class switching to IgE (**g**). IL-4, IL-13 and CD40L also stimulate the expression of CD23 and the release of soluble CD23 (**h**). In humans, soluble trimeric CD23 up regulates IgE synthesis and secretion through interaction with CD21 (**i**).*

1.8 Allergen dependent and independent mechanisms of mast cell activation

It is possible that IgE mediates mast cell activation and degranulation in the mucosa of non-atopic asthmatics by cryptic allergen dependent and independent mechanisms. In the former, allergen-specific IgE antibodies may be restricted to the bronchial mucosa and therefore not detected by the conventional skin or serological tests. Alternatively, allergen-independent mast cell activation by IgE may occur by way of the "cytokinergic" activity of certain IgE molecules or the activity of free immunoglobulin light-chains.

1.8.1"Cryptic allergen" dependent mechanisms

1.8.1.1 Common allergens:

Estimates of between 20 and 60% of rhinitis patients have IgE antibodies against common allergens confined to the nasal mucosa according to reports from different laboratories[135-138]. The "local IgE" exhibits a higher ratio of allergen-specific to total IgE than that in serum from the same individual[91]. Bronchial provocation of atopic asthmatics causes an increase in allergen-specific IgE in the airways, which is not detected in the circulation[139]. Together these observations strongly suggest local generation and sequestration of IgE antibodies to common allergens at least in atopic patients.

1.8.1.2 Rare allergens:

These are identified allergens but not amongst the small number of common ones tested in the routine diagnosis of atopic asthma. Examples are allergens such as egg, wheat, rye, soybean, α-amylase etc.[140,141].

1.8.1.3 Auto-allergens:

IgE antibodies directed against auto(self) allergens are observed in atopic dermatitis, predominantly in patients with severe and chronic disease[142]. Valenta *et al.* have determined their specificities by screening a phage expression library of human cDNAs with IgE from patient serum. The auto-antigens comprise a wide variety of proteins expressed in unrelated human cell types and tissues and the reactions in skin are attributed to local tissue damage. It is possible that auto-allergens may play a similar role in non-atopic asthma[143].

1.8.1.4 Anti-IgE or FcεRI auto-antibodies

These may mimic allergens by cross-linking IgE bound to FcεRI, or the receptor itself, on mast cells. Such antibodies (generally of the IgG class) induce urticaria by activating skin mast cells in about 30-40% of patients[143-145]. Tedeschi *et al*.[146,147] have shown that injection of autologous serum into the skin of a sub-group of non-atopic asthmatics caused a wheal and flare reaction. The serum also induced degranulation of basophils from normal donors *in vitro*. Anti-IgE or anti-FcεRI auto-antibodies may therefore be implicated in non-atopic asthma[148].

1.8.2 *Staphylococcus aureus* enterotoxins

Staphylococcus aureus is a commensal bacterium that colonises the nasal mucosa intermittently or permanently in some 60% and 20% of the population respectively[149]. It may secrete a range of more than 50 enterotoxins. The "classical" *S. aureus* enterotoxins A (SEA), B (SEB), C (SEC), D (SED), E (SEE) (up to U) and Toxic shock syndrome toxin (TSST-1) act as T cell and/or B cell superantigens which bind to relatively conserved framework regions of T and/or B cell receptors rather than to the complementarity-determining regions. Certain superantigens can therefore stimulate the proliferation of a vastly greater proportion

of B cells than ordinary antigens and cause a cytokine storm *in vivo*[150]. Selective amplification of the "cognate" immunoglobulin families results in characteristic changes in the immunoglobulin repertoire[151]. Such changes have been observed in the target organs of autoimmune and infectious diseases[151], allergic rhinitis[33,131,152] and asthma[100].

1.9 The concept of allergen-exacerbated, non-atopic asthma

Although "non-atopic" patients by definition lack IgE responses to recognised aeroallergens, it remains possible that the symptomatology, if not the pathogenesis of asthma might be driven by unidentified allergens as described in the previous sections, where the allergen cannot be detected in the periphery by skin prick or specific IgE testing. Patients with late-onset eosinophilic asthma are less often typically "allergic" by conventional criteria[5,153-155]. Nevertheless, as elaborated above, many of these patients have elevated levels of total IgE, and may have IgE responses directed against environmental proteins not conventionally regarded as allergens, such as Staphylococcal superantigens[156,157]. In addition, late-onset eosinophilic asthma is often associated with sensitivity to non-steroidal, anti-inflammatory medications (aspirin)[153,158].

Alarmin-driven mucosal inflammation in asthma may drive the development of IgE against a wide variety of local antigens, many of which are never looked for in conventional "allergy" tests. Thus the concept of dividing asthma into "atopic" and "non-atopic" on the basis of a few, arbitrary skin prick tests may not be entirely accurate. The question is precisely what role, if any, these IgE molecules play in the propagation of mucosal inflammation and symptomatology in asthma.

1.10 Novel allergen independent mechanisms

1.10.1 "Cytokinergic IgEs"

These are a subset of IgEs that activate mast cells in an allergen-independent manner. Their activities were recently discovered and elucidated by Kawakami *et al*. in mouse. There are also a small number of reports of this activity in human systems[159,160]. The "highly cytokinergic" murine IgE SPE7 is monomeric in solution but self-associates upon binding to FcεRI on mast cells, thereby activating the cells[161-163]. It has recently been demonstrated for the first time that approximately half of the recombinant IgEs derived from the nasal mucosa of allergic rhinitis patients are highly cytokinergic[164]. Cytokinergic activity requires relatively high concentrations of IgE (1-5 μg/ml), similar to the concentrations required for up-regulation of FcεRI, as present in the bronchial mucosa in both atopic and non-atopic asthma.

1.10.2 Free immunoglobulin light-chains

These are by products of high-rate immunoglobulin synthesis in B cells. Recent work by Redegeld *et al*. has shown that they induce mast cell activation *in vitro* and *in vivo* in a mouse model of asthma[165-168]. In addition, a 9-amino acid peptide antagonist (F991), which acts as a competitive inhibitor of light-chain binding to an unknown receptor on mast cells, is protective *in vivo* in the mice[169,170]. The authors suggest that the limited efficacy of omalizumab in atopic asthma, and the perplexing non-responsiveness of some 30% of all patients[171] , may be partly due to the activities of free light-chains. Omalizumab does not suppress IgE synthesis[172], and therefore synthesis of excess light-chain may continue or even increase when free IgE is largely neutralised.

The hypothesis is that IgE is implicated in the pathogenesis of non-atopic asthma by participating in one or more of above described mechanisms of chronic mast cell activation. My aim was to analyse some of the above mentioned conceivable mechanisms of mast cell activation involving IgE, in a group of atopic and non-atopic asthmatics and controls broadening the fundamental understanding of the potential role of IgE antibodies in non-atopic asthma and generating further hypotheses concerning novel diagnostic approaches and therapeutic strategies.

1.11 Omalizumab and its role in asthma treatment

Omalizumab is a humanised, monoclonal IgG_1 antibody which binds to the Cε3 constant domain of human IgE, blocking binding to both its high- and low-affinity receptors FcεRI and FcεRII (CD23)[124,126,173,174]. It does not cross-link cell surface-bound IgE, which might result in anaphylaxis. It forms complexes with free IgE in the circulation. Consequently, provided a sufficient dosage of omalizumab is given, free IgE is reduced in the circulation within a few days to barely detectable levels (<25 ng/ml). Since free IgE up-regulates the expression of its own receptor FcεRI, expression of this receptor on mast cells and basophils is reduced over a period of months, provided that regular therapy is continued[128].

The humanised, monoclonal anti-IgE antibody "omalizumab" is the first major new development in asthma therapy in nearly 10 years. It has already been used under licence to treat thousands of allergic asthmatics in many countries. It has been proven to reduce the frequency of exacerbations in severe allergic asthmatics, resulting in fewer unplanned hospital visits: a factor most detrimental of all to quality of life based on the results from previous clinical trials[175]. Its efficacy and safety are well documented[176].

1.12 Omalizumab clinical trials

i) INNOVATE trial[175]: This was one of the landmark trials to establish the therapeutic efficacy of omalizumab in allergic asthma. A total of 419 patients with severe persistent allergic asthma receiving GINA step 4 treatment were included in the study. The results revealed that in patients with inadequately controlled severe persistent asthma despite high-dosage ICS and LABA therapy, and often additional therapy, omalizumab significantly reduced the rate of clinically significant asthma exacerbations, severe exacerbations and emergency visits.

ii) In a similar randomized clinical trial[177], omalizumab was found to be efficacious in reducing the numbers of exacerbations suffered by patients with severe, persistent asthma during a steroid stable phase and a steroid reduction phase. The addition of omalizumab to standard asthma therapy reduced asthma exacerbations and decreased inhaled corticosteroid and rescue medication usage. Omalizumab was well tolerated, with an adverse events profile similar to that of placebo.

iii) The steroid sparing effect of omalizumab was further confirmed in a trial[178] where it reduced the mean exacerbation rate by 52% during a steroid reduction phase and by 58% during a subsequent steroid stable phase. These data indicated that omalizumab therapy safely improves asthma control in allergic asthmatics who remain symptomatic despite regular use of inhaled corticosteroids and simultaneous reduction in inhaled corticosteroid requirement.

iv) The efficacy, safety and steroid sparing effect of omalizumab in allergic asthma were further confirmed in another clinical trial[179] where therapy reduced the inhaled corticosteroid load of the patients with no deterioration in their symptomatology or increase in the use of rescue medication.

v) Another interesting and relevant argument gaining popularity lately is that omalizumab enhances anti-viral immunity by improving the IFN-α response to rhinovirus. Some patients

with asthma have reduced antiviral type I and III interferon responses, a defect also noted with peripheral blood plasmacytoid dendritic cells isolated from asthmatic patients and associated with IgE concentration on the cell surface[180-183]. In a randomised controlled trial of inner city children, omalizumab improved IFN-α responses to rhinovirus, and within the omalizumab group, greater IFN-α increases were associated with fewer exacerbations.[184]

Because of incomplete knowledge of how omalizumab works, it is currently denied to non-atopic asthmatics, who form about 30% of the total population of adult asthmatics and who are more likely to have disease uncontrollable by conventional therapy. By this study I wished to test for proof of principle that omalizumab is of therapeutic benefit to non-atopic asthmatics, which would open up this therapeutic option to approximately 30,000 more patients in the UK and many more worldwide.

1.13 Omalizumab in non-atopic asthma

Since omalizumab blocks IgE binding to its receptors, the tacit assumption, yet to be verified, is that it exerts its anti-asthma effect fundamentally by abolishing allergen-induced mast cell and basophil degranulation in atopic patients who manufacture allergen-specific IgE (in other words, it only works on atopic asthmatics). Consequently, to date, trials of its efficacy have been limited to severe atopic asthmatics with evidence of circulating allergen-specific IgE production as detected by one or more positive skin prick or *in vitro* tests. Its licence currently restricts its use to such patients. Given the possibility that non-atopic asthmatics also make allergen-specific IgE which is sequestered in the bronchial mucosa, and that many other IgE species not specific for allergens may play a role in asthma pathogenesis as discussed above, inhibition of allergen-induced mast cell degranulation may not be the only, or even the principal mechanism by which omalizumab alleviates asthma. Omalizumab administration

results in a substantial reduction of free IgE and consequent down regulation of FcεRI. Since epidemiological studies have shown that serum concentrations of IgE, regardless of its specificity, are directly associated with the risk of non-atopic asthma, a reduction of functionally active IgE may be postulated to be beneficial in non-atopic asthmatics[125]. Omalizumab therapy has also been shown to reduce bronchial mucosal inflammation in atopic asthmatics[171,185]. The mechanism of this effect (in particular if and how it is related to removal of IgE) is unknown, but the observation is important since it suggests that, given the aforementioned evidence, the strategy may be anti-inflammatory in non-atopic, as well as atopic asthma.

Consequently, I considered it of vital and pressing importance to investigate the therapeutic potential of anti-IgE therapy in non-atopic asthmatics. Omalizumab is developed and ready for use, and there is no reason for delay while patients continue to suffer. Its principal benefit, as mentioned, is in reducing unexpected disease exacerbations[186], in patients who continue to have these exacerbations despite maximal conventional therapy. Since it would require a large, lengthy and costly clinical trial to demonstrate benefit of omalizumab in terms of reduction of disease exacerbations in a group of severe non-atopic asthmatics taking maximal conventional therapy, I determined initially to conduct a proof of concept study powered to detect differences in clinical and physiological outcome measures between omalizumab and matched placebo therapy in severe, non-atopic asthmatics whose disease was provoked in the shorter term by reduction of therapy. I remain hopeful that this will provide sufficient impetus for more extensive clinical trials and alteration of omalizumab licensing. I also took the opportunity to measure possible effects of omalizumab on inflammatory cellular infiltration and IgE expression in the bronchial mucosa of non-atopic asthmatics.

I conducted a placebo controlled, double blind, parallel group study to test for proof of principle that omalizumab exerts beneficial effects on disease control in non-atopic moderate and severe adult asthmatics aged 18-75 years (British Thoracic Society treatment steps 3 and above). My aim was to recruit and randomise up to forty patients in a 1:1 ratio to receive omalizumab or matching placebo. Following 12 weeks of treatment with omalizumab or placebo, existing anti-asthma treatment was progressively reduced while omalizumab/placebo therapy was continued. Dosages of omalizumab or placebo were administered at 4 or 2 weekly intervals over a 16-week period (5 or 10 doses in total), which corresponded with the time stated as necessary to judge efficacy of therapy according to omalizumab's licensed indications in atopic asthma. Efficacy was judged by clinical monitoring and by bronchial biopsy to assess effects on bronchial inflammation and IgE expression.

1.14 Hypotheses of the project

- Allergen-specific IgE is expressed in the bronchial mucosa of non-atopic, as well as atopic asthmatics.
- In non-atopic asthmatics, IgE has the potential to activate target cells by allergen-dependent mechanisms involving allergen-specific IgE and by additional, independent mechanisms mediated by anti-IgE auto-antibody.
- Anti-IgE (omalizumab) therapy reduces bronchial mucosal IgE expression and inflammation, and improves lung function in the face of staged reduction of "standard" therapy in non-atopic asthma.

1. 15 Aims and objectives of the project

- To compare changes in lung function, disease control and quality of life as well as histological indices of inflammation and IgE expression in the bronchial mucosa of a group of moderate and severe, non-atopic asthmatics treated in a double-blinded fashion with omalizumab or placebo for the standard period of 16 weeks while "standard" anti-inflammatory therapy is reduced.
- To measure total and allergen-specific IgE in the bronchial mucosa of atopic and non-atopic asthmatics and non-atopic controls.
- To investigate the possible effects of anti-IgE autoantibody in promoting and inhibiting basophil (and putatively mast cell) activation.

Chapter 2: Materials and methods

2. Subjects, materials and methods

2.1 Subjects

Study subjects were identified from the asthma clinics, the departmental database or through advertisements. Atopic and non-atopic asthmatic patients and non-atopic non-asthmatic control subjects (non-atopic controls) were identified based on the definitions and criteria described below. Non-atopic asthmatics were also identified as part of the clinical trial "omalizumab in non-atopic asthma".

2.2 Definitions

2.2.1 Asthma

A diagnosis of asthma was made based on relevant symptoms and one of the following criteria

- Documented ≥ 12% reversibility of FEV1 or PEFR in response to inhaled bronchodilators (β-agonist and/or anticholinergics)
- Documented ≥ 20% variability of PEFR readings over a period of 1-2 weeks
- Documented history of positive bronchial challenge test

Non-asthma was defined as lifelong absence of relevant symptoms with FEV1 in the normal range.

2.2.2 Atopy

Atopy was defined, following worldwide clinical consensus, as positive skin prick test and/or *in vitro* IgE test to one or more of the following aeroallergens: mixed grass, mixed tree, mixed mould, house dust mite, and cat and dog dander.

2.2.3 Non-atopy

Non-atopy was defined as negative skin prick and *in vitro* IgE tests (Phadia Grade 0 or ≤ 0.35 kU/L) to the same common aeroallergens.

2.3 General exclusion criteria:

i) Age < 18 or > 75 years

ii) Asthmatics with FEV1 < 40% predicted at the time of study

iii) Patients taking >20mg of prednisolone daily

iv) Total smoking history ≥ 10 pack years

v) Subjects who are pregnant, at risk of pregnancy or lactating

vi) Subjects with any chronic pulmonary, cardiac or other disease which may compromise the safety of fibreoptic bronchoscopy or interpretability of the study

vii) Inability or unwillingness to co-operate with the clinical protocol.

2.4 "Omalizumab in non-atopic asthma"- brief summary of the clinical trial

This was a randomised, placebo-controlled, double blind, parallel-group, proof of concept trial of 20 weeks' duration. Omalizumab and identical vehicle control manufactured to GCP standards were kindly supplied by the manufacturers (Novartis Pharmaceuticals). The trial was approved and monitored by Guy's Research Ethics Committee (REC Ref: 09/H0804/43)

and the Medicines and Healthcare Products Regulatory Agency (CTA No: 14523/0219/001/0001) and registered on clinicaltrials.gov (reference NCT01113437). Eligible patients were moderate/severe, non-atopic asthmatics who provided written, informed consent recruited from the asthma clinics at Guy's and St. Thomas', the Royal Brompton and the Homerton University Hospitals in London, the departmental database or through advertisement.

Asthma and non-atopy were defined as described in the earlier paragraphs. Moderate/severe asthma was defined as regular (at least 3 days per week) day- and night-time symptoms in the 3 months prior to screening despite regular step 3-5 asthma treatment according to the BTS guidelines[187]. The non-atopic status of these participants was further confirmed by full ISAC (Phadia) screening of their sera and bronchial biopsy homogenates (data presented in chapter 4) [188].

Patients in the following categories were excluded from taking part in the trial: i) Smoking within 3 months prior to screening or total smoking history >10 pack years, ii) Pregnant or lactating females or those at risk of pregnancy, iii) Patients taking >20mg of prednisolone daily, iv) Hospitalisation for asthma or exacerbation requiring systemic corticosteroid therapy within 3 months of the screening visit, v) History of life-threatening asthma, defined as an asthma episode that required intubation and/or was associated with hypercapnia, respiratory arrest and/or hypoxic seizures within the 6 months prior to screening, vi) Pre-bronchodilator FEV1 < 40% predicted, vii) Patients in whom omalizumab therapy was conventionally contraindicated or should be used with caution according to the omalizumab SmPC.

The phases of the study protocol are summarised in **Figure 2.1.**

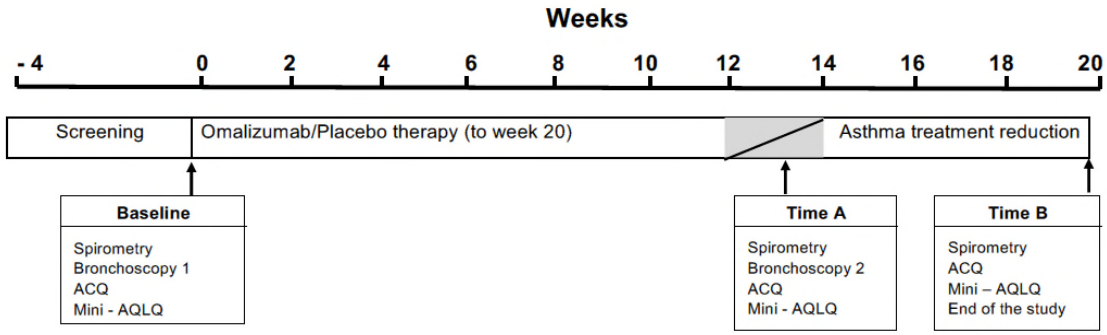


Figure 2.1: Clinical trial flow chart outlining interventions. **Baseline:** Time from screening visit to first bronchoscopy and commencement of omalizumab/placebo (Weeks -4 to 0). **Time A:** Time span during which the patients had a second bronchoscopy (Weeks 12 to 14) after which therapy was reduced. **Time B:** End of the trial 20 weeks from the first injection of omalizumab/placebo.

***Screening/baseline*:** After screening, during a baseline period of up to 4 weeks patients were given and instructed to use, if necessary, a portable peak flow meter (Mini-Wright Standard EU Scale, SKU: 3103387, Clement Clarke International Limited) and blank diary forms (Juniper Asthma Control Diary[189]) in which they documented daily morning and evening peak expiratory flow (PEF) and day and night symptoms (on a 0-6 scale) until the end of the study. Existing anti-asthma medication was not changed at this stage but compliance encouraged.

***First bronchoscopy and commencement of therapy*:** At a second visit patients completed a Juniper Asthma Control Questionnaire[189] (ACQ) and mini-Asthma Quality of Life

Questionnaire[190] (mini-AQLQ), then underwent pre-bronchodilator spirometry (Minispir® PC based Spirometer, Winspiro Pro version 4.1.5 software) prior to the obtaining of 10 technically suitable bronchial mucosal biopsies from the right or left second or third generation bronchi at fibreoptic bronchoscopy using an Olympus bronchoscope model BF XT40 OES. 50ml of venous blood were collected during this and the visit for the second bronchoscopy. Patients then received their first subcutaneous injection of the trial medication (omalizumab or identical placebo, allocated by the hospital pharmacy using randomisation tables with the patient and attending physician blinded), the dosage and frequency of which (either 2 or 4 weekly) were determined as in standard clinical practice based on their initial body weight and serum total IgE concentration as described in the Omalizumab SmPC. Where serum total IgE was below the lowest concentration in the SmPC dosing table the lowest dosage in the table (75 mg every four weeks) was administered. Patients were observed for 2 hours afterwards. At each subsequent dosing visit, patients were examined clinically, encouraged to comply with their usual medication and their diary cards collected and renewed.

***Second bronchoscopy*:** Within a 2-week window between 12 and 14 weeks after commencement of omalizumab/placebo therapy (**Time A,** Figure 2.1), lung function was re-measured and repeat bronchial biopsies obtained as before.

***Therapy reduction phase*:** Patients were instructed carefully how to use a Turbohaler® device and asked, commencing the day following the second bronchoscopy, to discontinue all existing inhaled and oral anti-leukotriene or theophylline based anti-asthma medications and substitute them with regular budesonide/formoterol combination therapy (Symbicort® 100/6 Turbohaler 2 puffs twice daily initially for 4 weeks and further reduced to 1 puff twice daily

until the end of the trial) with additional terbutaline (Bricanyl® Turbohaler 500 μg/puff) as required for immediate relief of symptoms. For patients taking regular additional oral prednisolone, an attempt was also made progressively to reduce the dosage according to a predetermined regimen depending on the dosage at entry to the study **(Table 2.1)**. Omalizumab/placebo therapy was continued for a total of 20 weeks while this new therapeutic regimen was pursued.

Starting dose of Prednisolone (mg)	Time A (12-14 weeks)	Week 16	Time B (20 weeks)
20	15	10	10
17.5	12.5	7.5	7.5
15	10	7.5	7.5
12.5	7.5	5	5
10	7.5	5	5
7.5	5	2.5	2.5
5	2.5	0	0

Table 2.1: Protocol for reduction in the dose of prednisolone

***End of the study (Time B/20 weeks, Figure 2.1)*:** At their penultimate visit, 20 weeks from commencement of omalizumab/placebo therapy, patients completed final ACQ and mini-AQLQ questionnaires then underwent repeat spirometry before being asked to resume their original anti-asthma therapy. A final visit was arranged 2 weeks later to check the patients' wellbeing and enquire about any adverse reactions.

At any time during the study, in the event of an asthma exacerbation, defined as a need for rescue oral corticosteroid medication for deterioration of symptoms and/or lung function, as

agreed between the patient and the study physician, patients were treated with a 10-day course of prednisolone 30 mg/day instituted by the study physician. Such patients left the study, resumed their regular anti-asthma medication and were followed up as necessary.

2.5 Clinical interventions for the mucosal IgE extraction and anti-IgE autoantibody experiments

Atopic and non-atopic asthmatic patients and non-atopic non-asthmatic control subjects (non-atopic controls) were recruited based on the definitions and criteria described in sections 2.2.1, 2 and 3. Non-atopic asthmatics were also identified as part of the clinical trial "omalizumab in non-atopic asthma". Flexible bronchoscopy was performed as described in sections 2.4 & 2.6.4, and ten technically suitable biopsies were collected. Three biopsies from each subject were weighed then snap frozen in liquid nitrogen for protein extraction and stored at -80 °C until analysed. The remaining biopsies were used for other related studies in the Department. All ten biopsies from some of the patients were weighed and homogenised for IgE extraction to detect possible biopsy-to-biopsy or site-to-site variability of IgE concentrations. 50 ml of venous blood were collected from each subject on the same day as bronchoscopy, allowed to clot on glass and centrifuged to isolate serum which was stored at -80°C until analysed.

2.6 Clinical interventions and assessment tools

2.6.1 Skin Prick testing

Standard skin prick tests were performed to investigate sensitisation to a full range of local aeroallergens. The participants were asked to discontinue any antihistamines they have been taking for at least 3 days prior to skin prick testing. Skin prick tests were performed on the volar aspect of the forearm using standard solutions (Diagenics/Allergopharma) such as tree

mix (early & mid-blossoming including alder, hazel, poplar, elm, willow, birch, beech, oak and plane), grass mix (velvet grass, cocksfoot, ryegrass, timothy, meadow grass and meadow fescue), house dust mite mix (*Dermatophagoides pteronyssinus* & *Dermatophagoides farinae*), mould mix (*Alternaria tenuis, Botrytis cinerea, Cladosporium herbarum, Curvularia lunata, Fusarium moniliforme, Helminthosporium halodes, Aspergillus fumigatus, Mucor mucedo, Penicillium notatum, Pullularia pullulans, Rhizopus nigricans, Serpula lacrymans*), cat epithelia and dog epithelia. The positive and negative controls were histamine (0.1%) and normal saline respectively. Tests were read after 15 minutes to look for a wheal and flare response. Any wheal 3 mm larger than the negative control in the presence of a clearly positive (histamine) control surrounded by a flare was defined as positive. Equivocal results were cross-checked and verified by looking for specific IgE antibodies in the serum using Phadia ImmunoCAP®.

2.6.2 Spirometry

Spirometry was performed using Minispir® PC based Spirometer (Winspiro Pro version 4.1. 5 software) according to the guidelines published by the American Thoracic Society and European Respiratory Societies. Bronchodilator reversibility was assessed after nebulisation with 2.5 mg of salbutamol and 500µg of ipratropium.

2.6.3 Mannitol Bronchial Challenge Test

Mannitol bronchial challenge test was performed using the Osmohale™ kit (Pharmaxis Pharmaceuticals Ltd, Buckinghamshire, UK), following the instructions from the manufacturer[191] and guidelines published by ATS/ERS task force[192]. Following withdrawal of relevant medications, a ≥15% fall in FEV1 from baseline or a ≥10% incremental fall between the mannitol dosages was considered to be a positive result. Bronchial challenge was not used

as a screening tool in the Omalizumab clinical trial to recruit moderate/severe non-atopic asthmatics.

2.6.4 Bronchoscopy

Flexible bronchoscopy was performed using an Olympus bronchoscope model BF XT40 OES in accordance with the departmental standard operating procedure. Ten technically suitable biopsies were collected using FB35C reusable or some similar non-reusable forceps from the right or left second and third generation bronchi.

2.7 Laboratory and in vitro procedures

2.7.1 Processing and storage of clinical samples

2.7.1.1 Bronchial biopsies

Bronchial biopsies were collected into separate small polystyrene containers (Bijou bottles) containing normal saline. Three biopsies were snap frozen in liquid nitrogen for protein (IgE) extraction, and stored at -80°C. Three biopsies were later processed for immunohistochemical and immunofluorescence experiments as described in sections 2.7.2, 2.7.2.1 and 2.7.2.2. The remaining biopsies were used for other projects within the Department.

2.7.1.2 PBMC isolation

The following steps were performed at room temperature:

40 ml of blood were diluted with an equal volume of sterile PBS and the mixture divided between two 50 ml Falcon tubes. 12.5 ml of Ficoll was placed into 50 ml falcon tubes (4 x for 80 mL diluted blood, 2x for 40 mL diluted blood). 20 ml of diluted blood were slowly and carefully layered onto 12.5 ml Ficoll inside the Falcon tube. The Falcon tubes were then centrifuged at 2000 rpm for 20 minutes at room temperature without braking. A total of 2 ml of plasma (top layer) was removed and aliquoted into cryovials. These were placed in a freezing container overnight before transferring to liquid nitrogen. Lymphocyte layers were removed and pooled into a new 50 ml Falcon tube and made up to 50 ml with PBS. The tubes were spun at 2000 rpm for 10 minutes. The cell pellet was re-suspended in approximately 10 ml of PBS. Falcon tubes were then topped up with PBS and centrifuged at 2000 rpm for 10min and the supernatant removed. The cells were then frozen in 10% DMSO (made with FCS) and placed in a freezing container overnight before transferring to liquid nitrogen. $1x10^7$ cells were prepared per cryovial.

Cell counting and viability

A 1:5 dilution was made with 20µL cell suspension from the pellet and 80µL of PBS. Cells were counted using a haemocytometer by adding an equal volume of trypan blue solution.

2.7.1.3 Serum preparation

A **t**otal of 10 ml blood from each patient were divided between two 5ml serum separating tubes (BD Vacutainer® SST™).

- Tubes were inverted several times immediately and then kept upright at room temperature for 30min (2hrs max).
- The tubes were then centrifuged for 10 minutes at 2000g to remove debris.
- 1ml of serum supernatant was aliquoted into cryovials and then placed in a freezing container overnight before transferring to liquid N_2.

2.7.2 Immunohistochemical techniques

Bronchial biopsies were processed and analysed using double immunofluorescence, single immunohistochemistry and confocal microscopy. Bronchial biopsies were fixed in 4% PFA for 2 hours at room temperature, washed overnight in PBS containing 15% sucrose, embedded in O.C.T. compound (Tissue-Tek®, VWR International LLC) and stored at -80°C. Eight micron thick sections were cut using a cryostat (Bright OTF 5000, Bright Instruments Co Ltd), air dried overnight onto Polysine slides (Thermo Scientific) and stored at -80^0C.

2.7.2.1 Immunofluorescence

PFA fixed sections were thawed at room temperature for 30 minutes and rehydrated in PBS for 5 minutes. Total tryptase$^+$ mast cells, CD20$^+$ B cells and CD138$^+$ plasma cells, CD38$^+$ plasmablasts, IgE$^+$ cells and cells of these phenotypes co-expressing IgE were sought and enumerated from digital images of the sections by two observers ignorant of the origin of the sections, using single and double immunofluorescence and confocal microscopy. Sections (18 sections per subject: 9 at baseline and 9 at time A) were stained with primary antibodies diluted in PBS containing 1% normal human serum and 1% normal goat serum (antibody buffer) or PBS/0.1% saponin for anti-CD20cy [mouse anti-CD138 (1:50; Dako), mouse anti-human CD38 (1:100, Abgent), mouse anti-human tryptase (1:100, Dako), mouse anti-human CD20cy (1:120, Dako), and rabbit anti-IgE (1:500, Dako)] and incubated overnight at room temperature. Excess antibodies were washed off in PBS (5 min X 3 times), then the sections incubated with anti-mouse-IgG-FITC (1:200; Life Technologies) and anti-rabbit-IgG-Alexa Fluor 594 (1:200; Life Technologies) diluted in antibody buffer for 1 hour at room temperature in the dark. After washing with PBS, the sections were mounted with Prolong Gold Antifade Reagent with Dapi blue nucleic acid background stain (Life Technologies). The images were captured using a Nikon Eclipse Ti-E inverted confocal microscope (Nikon Imaging Centre, King's College London). The images were processed under identical conditions of illumination using ImageJ (http://rsb.info.nih.gov/ij/) and the total numbers of positively stained cells (green fluorescing CD20$^+$, CD138$^+$ and tryptase$^+$ cells and red fluorescing plasmablasts, IgE$^+$ cells and those expressing both red and green fluorescence measured confocally) counted and expressed as per mm^2 of the entire area of the submucosa. The typical mean co-efficient of variability between the sections was 67.46% for tryptase$^+$ mast cells and 93.01% for CD138$^+$ plasma cells.

2.7.2.2 Immunohistochemistry

Bronchial biopsies were fixed, frozen and stored as described in the immunofluorescence section. Total BMK-13^{+} cells were sought and enumerated using single immunohistochemistry and confocal microscopy. Sections were stained with BMK-13 (1:30, Abcam) diluted in PBS containing 5% normal human serum and incubated overnight at room temperature. Excess antibodies were washed off in PBS (5 min x 3 times), then the sections incubated with rabbit-anti mouse secondary antibody (1:50, Dako) in PBS containing 5% normal human serum for 30 minutes at room temperature in a humidifier chamber. After washing with PBS, the sections were incubated with mouse - APAAP (1:30, AbD Serotec) in PBS containing 5% normal human serum for 30 minutes at room temperature and then washed again with PBS (5 min x 3 times). Fast Red solution, prepared by dissolving Fast Red tablets (Sigma) in dH_2O, was applied to the sections for 20 minutes or until a suitable colour change was observed under the microscope. The Fast Red reaction was stopped with H_2O for 5 minutes. Haematoxylin was applied for 30 seconds, and then rinsed off under running water for 5 minutes. The slides were then dried for an hour, mounted with Glycerol Gel (Dako) and dried overnight. The images were captured using Nikon Eclipse Ti-E inverted confocal microscope (Nikon Imaging Centre, King's College London). The images were processed under identical conditions of illumination using ImageJ (http://rsb.info.nih.gov/ij/) and the total numbers of BMK-13 positively stained cells (red colour) counted and expressed as per mm^2 of the entire area of the submucosa.

Primary, secondary and tertiary antibodies used in the immunohistochemical and immunofluorescence experiments, their respective dilutions, source and catalogue numbers are listed in the table 2.2.

Table 2.2

Immunofluorescence Experiments			
Antibody	**Source**	**Dilution**	**Origin/Catalogue no.**
Primary Antibodies			
Monoclonal Anti-Human Mast cell Tryptase	Mouse	1:100	Dako, M7052
Monoclonal Anti-Human CD138 (Plasma cells)	Mouse	1:50	Dako, M7228
Monoclonal Anti-Human CD20cy (B cells)	Mouse	1:120	Dako, M0755
Monoclonal Anti-Human CD38	Mouse	1:100	Dako, M7254
Polyclonal Anti-Human IgE	Rabbit	1:500	Dako, A0094
Secondary Antibodies			
Fluorescein F(ab')2 Fragment of Anti-Mouse IgG (H+L)	Goat	1:200	Life Tech, F11021
Alexa Fluor® 594 Anti-Rabbit IgG (H+L) Antibody	Goat	1:200	Life Tech, A11012

Immunohistochemistry Experiments			
Primary Antibodies			
Monoclonal Anti-Human Mast cell Tryptase	Mouse	1:100	Dako, M7052
Monoclonal [BMK13] to PRG2	Mouse	1:30	Abcam, AB77842
Monoclonal Anti-Human CD138 (Plasma cells)	Mouse	1:50	Dako, M7228
Monoclonal Anti-Human CD20cy (B cells)	Mouse	1:120	Dako, M0755
Secondary Antibody			
Anti-mouse immunoglobulins, absorbed	Rabbit	1:50	Dako, Z025902-2
Tertiary Antibody			
APAAP Complex	Mouse	1:30	AbD Serotec, STAR67

2.7.3 Protein extraction from bronchial biopsies

The purpose of these experiments was to attempt directly to measure bronchial mucosal total and allergen specific IgE.

2.7.3.1 Homogenisation of bronchial mucosal biopsy samples

The biopsies were weighed before homogenisation. The frozen biopsies were pulverized into a fine powder using a commercial cell crusher (Figure 2.2; Cell crusher/homogeniser, Stratech Scientific UK Ltd). The powdered tissue was then dispersed in ice cold lysis buffer (30 µl/mg of tissue). This was prepared by dissolving 0.48 g of TRIS (Sigma Aldrich®) and 0.877 g of NaCl (Sigma Aldrich®) in 70 ml of distilled water and then adjusting the pH to 7.5 by adding hydrochloric acid. 1ml of 0.5M EDTA (Sigma Aldrich®) and 1g of CHAPS (Thermo Fisher Scientific Inc., IL, USA) were added to the above solution and the final volume made up to 100 ml by adding more distilled water. Protease inhibitor cocktail (100X, Pierce Thermo scientific) was added to the lysis buffer mixture at a volume ratio of 1:100. The suspensions were vortexed for 30 seconds then homogenised with a Sigma handheld homogeniser (10 times) and incubated on ice for 20 min. The lysed suspensions were then homogenised again (10 times) then incubated again on ice for 20 min. Finally, they were sonicated in a water bath for four 5 second periods separated by 30 second intervals then incubated on ice for 20 min. They were finally centrifuged at 13000 rpm at 4^0C for 30 minutes, then transferred (diluted, if appropriate depending on the final volume of the homogenate and the volume required for the assay, in lysis buffer without CHAPS) into separate clean tubes.

Figure 2.2: Cell crusher/homogeniser- Stratech Scientific Ltd

Prior to analysis, CHAPS detergent was removed from the samples using Pierce detergent removal spin columns (Thermo Fisher Scientific Inc., IL, USA). The homogenates (about 200 µl) were finally concentrated by ultra-filtration using Vivaspin2 spin columns (Molecular weight cut-off 3 KDa, Sartorius Stedim Biotech, UK Ltd, 500 µL-2 ml) and centrifuged at 4000 rpm for 20 min producing a final volume of approximately 50 µl. Concentrated homogenates were aliquoted and stored on ice for immediate analysis or at -80 °C for later analysis.

2.7.3.2 Total IgE estimation by ImmunoCAP 100

ImmunoCAP provides an *in vitro* test for quantitative measurement of the total amount of circulating IgE in human serum or plasma samples. In addition, we developed the technique to measure IgE in tissue homogenates in this project.

2.7.3.2.1 Test Principle

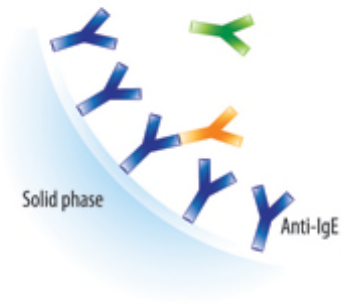
The technology is based on provision of an extremely high total binding capacity on a cellulose substrate. This ensures binding of all relevant antibodies, regardless of affinity while minimising non-specific binding. The ImmunoCAP solid phase consists of a cellulose

derivative enclosed in a capsule. The hydrophilic, highly branched polymer provides an ideal microenvironment for antigen/antibody interactions. The protocol was designed as a sandwich immunoassay as outlined in Figure 2.3.

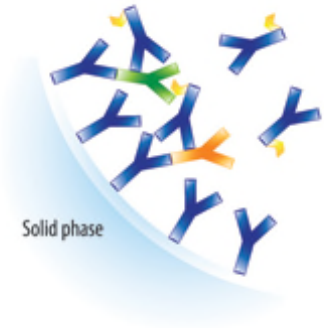
2.7.3.2.2 ASSAY PROCEDURE

The IDM (ImmunoCAP 100^{E}) machine and associated software were started and the total IgE assay run according to the manufacturer's instructions. (A detailed standard operating procedure for this assay is included in Appendix1. The steps are outlined below).

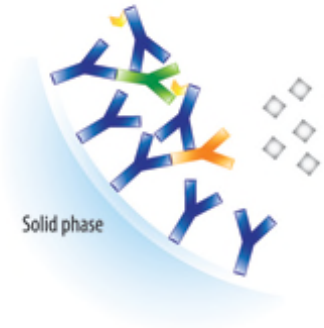
The caps were removed on the reagents and samples before placing them in the sample carousel. It was ensured that there was sufficient volume of samples and reagents to be drawn into the machine. Air bubbles or film on the surface of the reagents/samples were removed as otherwise the pipette sensor will not detect the surface properly. Washing, rinse and waste bottles were also loaded beforehand. About 50µL of each sample were loaded and placed on the loading slots as the carousel moved to the next position. All the required controls were loaded. Then all the required reagents (i.e. total IgE conjugate, development solution, stop solution, etc.) were added in the given positions. The next step was to start loading the ImmunoCAP /EliA Wells and a blank check. After the blank check was completed, the appropriate ImmunoCAP /EliA Wells were loaded in the dispenser when requested. The Lot Specific Code found on the label on the EliA Well foil bag and on the EliA Well Carrier was entered. When all ImmunoCAP/EliA Wells were loaded, the carrier was removed and the assay run process started. The IDM machine is configured to run automatically when primed.



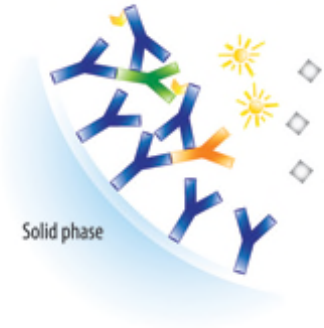
Anti-IgE, covalently coupled to the solid phase, reacts with the total IgE in the patient serum sample



After washing the second layer, enzyme-conjugated anti-IgE antibodies are added to form a complex.



After incubation, unbound enzyme-labelled anti-IgE is eluted and the bound complex incubated with a developing agent.



After stopping the reaction, the fluorescence of the developed reagent is measured. The fluorescence is proportional to the concentration of IgE in the serum sample and calibrated using a reference sample.

Figure 2.3: ImmunoCAP for total IgE - test principle

2.7.3.3 Measurement of specific IgE to multiple allergen components using Phadia ImmunoCAP ISAC® Micro Array

2.7.3.3.1 ImmunoCAP ISAC (Immuno Solid Phase Allergen Chip) is a diagnostic test using biochip technology. It allows simultaneous measurement of specific IgE antibodies to multiple allergen components in a single test, using only as little as 20 µl of the biological sample. The specific IgE chip delivers results for over a hundred components from more than 50 allergen sources. The allergens are pre-selected and include the most relevant species specific and cross-reactive markers but not necessarily every allergen component to which the patient may be sensitised.

2.7.3.3.2 Principles of the procedure

ImmunoCAP ISAC is a solid-phase immunoassay. Allergen components immobilised on a solid substrate in a microarray format are incubated with human biological samples to detect specific IgE antibodies. Binding of the specific IgE antibodies to the immobilised allergen components is detected by the addition of a secondary fluorescence-labelled anti-human IgE antibody. The procedure is followed by image acquisition using an appropriate microarray scanner. The ISAC Standardized Units for specific IgE (ISU) are determined and the test results analysed with proprietary software (MIA - Microarray Image Analysis Software). An outline of the test is presented in Figure 2.4 and detailed protocol in Appendix 2.

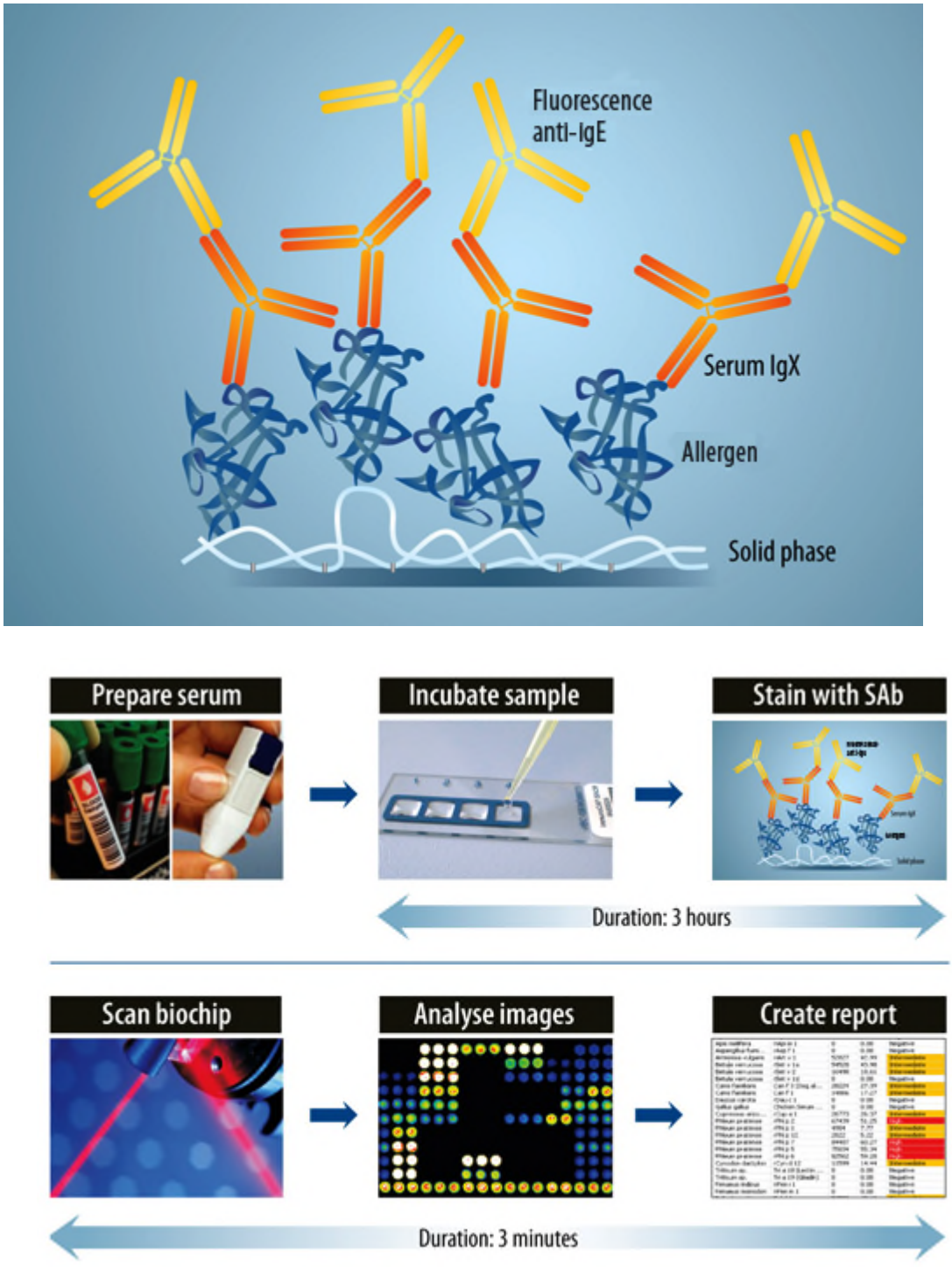


Figure 2.4: ImmunoCAP ISAC test outline

2.7.3.3.3 Reagents

ImmunoCAP ISAC Assay Kit IgE and ImmunoCAP ISAC Starter Kit IgE contain the reagents required to perform the assay. (More under protocol later: Appendix 2).

2.7.3.3.4 Specimens

Sera and bronchial biopsy homogenates from the participants were analysed. Blood samples were collected using standard procedures. The biopsy homogenates were concentrated using Vivaspin-2 (MWCO 3000). Specimens were stored at 2 – 8°C for up to one week or at –20°C for prolonged periods. Repeated freezing and thawing were avoided.

2.7.3.3.5 Protocol

35 ml of component A were added to 665 ml of purified water to prepare 700 ml of freshly prepared solution A (1:20 dilution). This volume was calculated for 3 washing steps of 220 ml each, using the washing dishes provided with the starter kit. A fresh paper towel was placed at the bottom of the humidity chamber and soaked with purified water. The lid of the humidity chamber was closed until further use to prevent evaporation. ImmunoCAP ISAC chips were placed in the washing dish containing the removable glass slide rack and approximately 220 ml of solution A together with a magnetic stirring bar and the solution stirred vigorously for 60 minutes on a magnetic stirrer. The glass slides containing the ImmunoCAP ISAC were moved into another washing dish containing approximately 220ml of purified water and stirred vigorously again for 5 minutes. They were then placed on a paper towel and left to air dry completely. The prepared ImmunoCAP ISAC slides were then placed in the humidity chamber with the reaction sites facing upwards. 20 µL of samples were pipetted onto each of the reaction sites (4 reaction sites per slide) while avoiding any direct contact of the pipette tip with the surface of the slides. The slides were then carefully placed in the humidity chamber

without mixing the samples and the lid closed. Incubation was allowed to proceed at room temperature for 120 minutes. The slides were removed from the humidity chamber carefully, without mixing the samples. The residual fluid was removed by tapping the chip with its long side on a fresh paper towel, taking care to avoid samples running over the neighbouring reaction sites. The ISAC chips were then washed in 220 ml of solution A for 10 minutes followed by 220 ml purified water for 5 minutes using the washing dish and magnetic stirrer as described previously. The washed slides were allowed to air dry completely and then placed in the humidity chamber with the reaction sites facing upwards. All used washing solutions were discarded. 20 µL of the IgE detection antibody solution were then pipetted onto each reaction site of the ImmunoCAP ISAC. ISAC slides were then replaced in the humidity chamber and incubated at room temperature for 60 minutes with lid closed to protect from light. The slides were then removed from the humidity chamber carefully and the IgE detection antibody solution removed by tapping the slide with its long side on a fresh paper towel or rinsing gently under distilled water. The slides were then washed in approximately 220 ml solution A for 10 minutes and then in 220 ml of distilled water for 5 minutes, using the washing dish and magnetic stirrer. All used washing solutions were discarded. The washed slides were allowed to air dry completely and thus become ready for reading. They were directly read in an appropriate microarray scanner or stored dry and protected from light for subsequent reading.

2.7.3.3.6 IgE recovery during extraction

In order to determine the recovery of IgE during the homogenisation and extraction processes, total and allergen specific IgE estimations were performed using the same assays (ImmunoCAP and ISAC) at each step of the extraction on tissue samples (and sera) from

some of the subjects. The IgE extraction experiments were also repeated using IgE standards of known concentrations and Phadia ISAC IgE control antibody at each step.

2.7.3.3.7 Variability between biopsies

In order to estimate the variability of mucosal IgE concentrations between different sites in the bronchial tree, total IgE was measured in 10 separate biopsies taken from the upper, middle and lower 1st -3rd generation bronchi from a single atopic asthmatic subject as described above.

2.7.4 Anti-IgE autoantibody experiments

Atopic asthmatic (AA), non-atopic asthmatic (NAA) and non-atopic non-asthmatic control (NAC) subjects were recruited as described in sections 2.2 and 2.3. Serum separation and PBMC isolation were performed as described in sections 2.7.1.2 and 2.7.1.3. All participant sera were screened for anti-nuclear autoantibodies (ANA) using commercially available ANA-ELISA kits (Abnova, Taiwan) according to the manufacturer's instructions.

2.7.4.1 ELISA

Maxisorp plates (Thermo Fisher Scientific) were coated either with 0.5 μg/mL of recombinant IgE (Abcam, Cambridge, UK) to measure IgG anti-IgE, or anti-human IgG (diluted 1:1000; AbD Serotec, Oxford, UK) to measure total IgG, in 50mM carbonate buffer pH 9.6 at 4°C overnight. Non-specific binding was blocked with SuperBlock blocking buffer (Thermo Fisher Scientific) or 3% milk powder (MP) in PBS-T at room temperature (RT) for 2 hours, and the plates then washed three times with PBS-T. Test sera were added at 1:5 to 1:20 dilutions in 1% MP-PBS-T and the plates incubated at RT for 1 hour, then washed three times with PBS-T and incubated with anti-human IgG-HRP (diluted 1:10000 in 1% MP-PBS-T;

Sigma-Aldrich, MO, USA) for 1 hour at RT. The plates were washed again 3 times and the colour reaction developed using TMB solution (R&D Systems). The reaction was stopped by addition of 1.8M H_2SO_4 and absorbance read at 450 nm using a Multiskan EX plate reader (Thermo Fisher Scientific). The assay was calibrated using a commercial IgG anti-IgE monoclonal antibody (omalizumab; Novartis, UK) or human IgG (Sigma-Aldrich). All samples were measured at least in duplicate. To further allow for any non-specific IgG binding to the plates and determine the threshold of sensitivity of the anti-IgE ELISA (0.27 ng/mL), commercial human IgG (Sigma-Aldrich) was used at a relatively high concentration (2 μg/mL) in place of sera as a negative control and the threshold defined as 3 standard deviations above the mean resulting absorbance.

The binding of IgG anti-IgE autoantibodies to FcεRI-bound IgE was determined by pre-coating the ELISA plate with recombinant FcεRIα (0.5 μg/mL; R&D Systems) in carbonate buffer at 4°C overnight. After washing 3 times with PBS-T and blocking with 3% MP in PBS-T for 2 hours at RT, the plates were incubated overnight at 4°C with recombinant IgE (0.5 μg/mL in 1% MP in PBS-T). Plates were washed 3 times with PBS-T before the addition of the test sera and the assay developed as described above. Each sample was assayed at least in duplicate.

2.7.4.2 Basophil activation test (BAT) by flow cytometry

Peripheral blood mononuclear cells (PBMC) from a single atopic non-asthmatic donor with total serum IgE > 150 IU/mL and sensitised to house dust mite as determined by skin prick testing were isolated using Ficoll-Paque PLUS (GE Healthcare) and re-suspended in HBSS (Sigma-Aldrich) to a final concentration of $2x10^7$ cells/mL.

To test the activity of subject sera in the BAT assay, $1x10^6$ PBMC (50 μL of suspension) were incubated with sera diluted 1:2 in BAT buffer to 50 μL (HBSS with 2mM $CaCl_2$) for 30 min at 37°C and the reaction stopped by adding 2 mL of FACS buffer containing 2mM EDTA on ice. The donor's own serum was used as a baseline control. As a positive control, instead of sera, PBMC were activated using polyclonal anti-IgE (Dako, Glostrup, Denmark) in the concentration range 6 ng/mL to 6 μg/mL in BAT buffer.

Flow cytometric analysis of basophil activation was adapted from a previously published protocol[193]. PBMC were stained with anti-CD203c-phycoerythrin (Clone NP4D6; BioLegend, CA, USA) and anti-CD63-allophycocyanin (Clone MEM-259; BioLegend) using the manufacturer's recommended concentrations in FACS buffer (PBS containing 2% FBS) for 30 min on ice, washed in FACS buffer and analysed using a BD FACSCalibur (BD Biosciences). Basophil activation was defined as the percentage of CD203c+ basophils expressing the activation marker CD63 compared with the baseline control.

2.7.4.3 Allergen-induced basophil activation

To determine the effect of subjects' sera on allergen-induced basophil activation, we pre-incubated 10^6 PBMCs in 50 μL of BAT buffer from a single atopic donor sensitised to house dust mite with the sera (diluted 1:2 in BAT buffer to 50 μL), purified anti-IgE, or the donor's own serum as a baseline control for 30 min at 37°C. The cells were then washed and resuspended in 50 μL BAT buffer prior to the addition of serial dilutions of the major HDM antigen *Der p2* from 300 to 3 ng/mL for 30 min at 37°C. The reactions were stopped by adding 2 mL of FACS buffer containing 2mM EDTA on ice, and basophil activation determined as described above.

2.7.4.4 Depletion of total IgG and IgE binding proteins from sera

To deplete total IgG from sera, 500 µL samples were incubated with an equal volume of protein G Sepharose (Sigma-Aldrich) at 4°C overnight in a mini Bio-Spin chromatography column (Bio-Rad, CA, USA). The flow through was recovered and re-incubated with fresh protein G Sepharose at 4°C overnight. IgG was measured in the final recovered flow through by ELISA.

To deplete IgE-binding proteins, IgE was cross-linked to cyanogen bromide (CNBr)-activated Sepharose 4B (Sigma-Aldrich) according to the manufacturer's instructions. Briefly, 100 µg of recombinant IgE anti-NP-BSA (manufactured in-house) was mixed with 100 µL of 1mM HCl-swollen CNBr-activated Sepharose in coupling buffer (0.1M $NaHCO_3$ buffer containing 0.5M NaCl) at 4°C overnight a mini Bio-Spin chromatography column, then washed with coupling buffer. Unconjugated sites were blocked with 0.2 M glycine buffer pH 8.0 for 2 hours at RT. After extensive washing with 5 cycles of the coupling buffer and 0.1M acetate buffer pH 4, the IgE-coupled Sepharose was ready to be used. IgE-coupled Sepharose (50 µL) was mixed with subjects' sera (3 mL) at 4°C overnight. The eluate containing non-IgE binding antibodies was stored at -20°C for later analysis. Proteins bound to the IgE-coupled Sepharose were then eluted with 100 µL of 0.2M glycine pH 2.5 into an equal volume of 100mM Tris buffer pH 8.0. The eluted solutions were dialysed in PBS at 4°C overnight, then mixed with protein G Sepharose (1:1) at 4°C overnight to capture IgG antibodies. The IgG anti-IgE antibodies were eluted off the protein G Sepharose with glycine, dialysed with PBS as above then quantified by ELISA and run on 10% SDS-PAGE under non-reducing conditions compared with recombinant IgE and IgG (Sigma-Aldrich). No contaminating IgE was found in purified antibodies as determined by total IgE ELISA (Figure 5.6, individual data not shown).

2.7.4.5 Effects of sera on allergen binding to IgE-bound basophils

To determine the effects of our test sera on allergen binding to IgE on the surface of basophils, we used pure, recombinant *Phl p7*-specific IgE sub cloned from an existing IgG_4 clone isolated in house[194] by replacement of the Cγ4 constant region with that of IgE[195]. Cells of the RBL-SX38 rat basophilic cell line, which stably express human FcεRI[196], were cultured in RPMI-1640 medium (Sigma-Aldrich) containing 10% FBS (Sigma-Aldrich), 1% Penicillin-Streptomycin-Glutamine (Life Technologies, CA, USA) and 1 mg/mL Genticin (Life Technologies). RBL-SX38 cells were harvested and resuspended in BAT buffer at $2x10^7$ cells/mL then incubated with *Phl p7*-specific IgE at a concentration of 0.5 µg/mL in 50 µL BAT buffer for 30 min at 37°C. Aliquots of 10^6 cells were washed with HBSS and incubated with 50 µL aliquots of the sera (diluted 1:2 in BAT buffer) for 30 min at 37°C. After further washing with HBSS, the cells were resuspended and incubated with 50 µL of 1 µg/mL of in house produced, freshly biotinylated *Phl p7* diluted in BAT buffer for 30 min at 37°C. Surface bound IgE was detected using anti-IgE-FITC (Vector Laboratories, CA, USA) and *Phl p7* binding to this IgE using streptavidin-APC (BioLegend), with analysis by flow cytometry.

Chapter 3: Effects of omalizumab on lung function, bronchial mucosal inflammation and IgE expression in non-atopic asthma

3.1 Introduction

Asthma remains a leading cause of suffering affecting about 300 million people worldwide[197]. In the UK, 10% of the 5.2 million sufferers are estimated to retain daily symptoms and remain vulnerable to acute exacerbations despite the regular and efficient delivery of conventional anti-asthma therapy, including systemic corticosteroid[198].

The humanised, monoclonal IgG_1 anti-IgE antibody omalizumab is the vanguard of what will hopefully form an arsenal of new biologicals able to improve the lives of severe asthma sufferers. That omalizumab therapy stabilises asthma control, reducing disease exacerbations, and consequently unplanned hospital admissions and exposure to systemic corticosteroid therapy in a substantial proportion of severe asthmatics is now acknowledged by professional and regulatory authorities worldwide, including the British Thoracic Society (BTS) and NICE[199] in the UK.

A major challenge when deploying treatment with biologicals for severe asthma is the possibility of mechanistic variation in the disease, requiring pre-identification of potential responders to any specific agent. This has generated intense interest in identifying "phenotypes" or "endotypes" of asthma. In the case of omalizumab, the *prima facie* effect of which is to prevent, and possibly reverse binding of IgE to its high- and low-affinity receptors[200,201], the tacit assumption has been that it improves asthma stability fundamentally by reducing or abolishing mast cell and basophil activation by cross-linking of surface-bound allergen-specific IgE by allergen in suitably sensitised, "atopic" patients. Consequently, key clinical trials investigating its efficacy, such the INNOVATE study[175] have been limited to atopic asthmatics while its marketing authorisation restricts its use to patients with "convincing IgE-mediated asthma". This phrase is not universally defined but is in practice

usually equated with evidence of IgE sensitisation (by skin prick or *in vitro* testing) to one or more common perennial aeroallergens. Conversely, the therapy has been denied to at least, we estimate, 20,000 otherwise eligible non-atopic severe asthmatics in the UK, and many more worldwide (the prevalence of non-atopic, severe asthma was estimated at 50% of the total in the ENFUMOSA cross sectional study[27] and 17–34% in the SARP study)[18].

Much indirect evidence suggests that IgE may play a role in asthma regardless of conventional atopic status. Epidemiologically, asthma was 5 fold more prevalent in a cohort of non-atopic subjects with elevated total serum IgE[125]. Many studies have shown that atopic and non-atopic asthma are virtually identical in terms of their bronchial mucosal cellular and molecular immunopathology[118-124,202], evidence of local B cell switching to IgE synthesis[53,203], elevated local FcεRI receptor expression[39] (local IgE up regulates FcεRI on expressing cells) and, very recently, elevated total bronchial mucosal IgE concentrations[188]. Furthermore, there is ample evidence that IgE directed against antigens other than aeroallergens, such as viral antigens[130] and Staphylococcal enterotoxins[204] which also act as superantigens, may play a role in asthma pathogenesis. IgE may influence the functions of mast cells by antigen-independent mechanisms[133,205]. Finally, IgE may exacerbate asthmatic bronchial mucosal inflammation by mechanisms other than causing degranulation of mast cells and basophils, such as by enhancing antigen capture by antigen-presenting cells and activating monocyte/macrophages[206]. Some patients with asthma have impaired, innate antiviral type I and III interferon responses, a defect also noted with peripheral blood plasmacytoid dendritic cells isolated from asthmatic patients and associated with IgE cross-linking on the cell surface[180-183]. In a randomised controlled trial of treatment of asthma in inner city children, omalizumab compared with placebo improved IFN-α responses to rhinovirus, which was associated in turn with fewer exacerbations[184]. Therefore, one of the fundamental effects of

anti-IgE therapy may be to restore, or at least greatly improve anti-viral immunity by reducing IgE-mediated inhibition of immune interferon α and β production by innate DCs in the respiratory epithelium, thus reducing damage to the epithelium, local alarmin production and up-regulation of Th2-type immunity, leading potentially to further IgE synthesis and a "vicious cycle" of increased IgE further impairing anti-viral defence. Presumably all of this is completely independent of conventional atopic status.

All of these data lend weight to the view that the presence or absence of atopy as operationally defined might not be an appropriate phenotypic or endotypic criterion for predicting responsiveness to omalizumab therapy.

To address this, I hypothesised that omalizumab therapy provides clinical benefit in chronic, severe asthmatics designated non-atopic by conventional criteria. Rather than embarking on a large, lengthy and costly clinical trial with frequency of exacerbations as a primary outcome measure, I elected in the first instance to provide proof of concept in a double-blind, placebo controlled study that omalizumab therapy can maintain or improve lung function in these patients despite provocation of the disease in the shorter term by supervised reduction of therapy. I also sampled their bronchial mucosa at fibreoptic bronchoscopy before and after therapy to address the hypothesis that omalizumab reduces local expression of IgE as well as the numbers of B cells, plasma cells and mast cells.

3.2 Subjects, materials and methods

3.2.1 Subjects

Eligible patients with moderate/severe, non-atopic asthma who provided written, informed consent were recruited from the asthma clinics at Guy's and St. Thomas' Hospital, with the

intention of supplementing this recruitment through collaboration with physicians in the asthma clinics at the Royal Brompton and the Homerton University Hospitals in London, and also from the departmental database or through advertisement.

3.2.2 Definitions

3.2.2.1 Asthma

A physician, based on relevant symptoms and one of the following criteria, made the diagnosis of asthma:

- Documented ≥ 12% reversibility of FEV1 or PEFR in response to inhaled bronchodilators (β-agonist and/or anticholinergics)
- Documented ≥ 20% PEFR variability over a period of 1-2 weeks

Moderate/severe asthma was defined as physician diagnosed asthma with regular (at least 3 days per week) daytime and night-time symptoms in the 3 months prior to screening despite taking inhaled and/or oral corticosteroid with or without long-acting β2-agonist or leukotriene blockers (regular step 3-5 asthma treatment according to the BTS guidelines[187]).

3.2.2.2 Non-atopy

Non-atopy was defined as negative skin prick and/or *in vitro* IgE tests (Phadia Grade 0 or ≤ 0.35 kU/L) to the following aeroallergens: mixed grass, mixed tree, mixed mould, house dust mite, and cat and dog dander.

The non-atopic status of these participants was further confirmed by full ISAC (Phadia) screening of their sera and bronchial biopsy homogenates (data presented in chapter 4) [188].

3.2.3 Inclusion criteria

i) Males and females aged 18 to 75 years inclusive.

ii) Moderate/severe asthma as defined above treated with inhaled corticosteroids, with or without oral corticosteroids (oral up to 20mg/day prednisolone or equivalent) for at least 3 months prior to screening.

iii) Written, informed consent provided.

3.2.4 Exclusion criteria

i) Age < 18 or > 75 years.

ii) Total smoking history >10 pack years.

iii) Pregnant or lactating females or those at risk of pregnancy.

iv) Patients taking >20mg of prednisolone daily.

v) Hospitalisation for asthma or exacerbation requiring systemic corticosteroid therapy within 3 months of the screening visit.

vi) History of life-threatening asthma, defined as an asthma episode that required intubation and/or was associated with hypercapnia, respiratory arrest and/or hypoxic seizures within the 6 months prior to screening.

vii) Pre-bronchodilator FEV1 < 40% predicted.

viii) Patients in whom omalizumab therapy was contraindicated or used only "with caution" according to the omalizumab SmPC.

3.3 "Omalizumab in non-atopic asthma"- clinical trial overview

This was a randomised, placebo-controlled, double blind, parallel-group, proof of concept trial of 20 weeks' duration. Omalizumab and identical vehicle control manufactured to GCP standards were kindly supplied by the manufacturers (Novartis Pharmaceuticals). The trial

was approved and monitored by Guy's Research Ethics Committee and the Medicines and Healthcare Products Regulatory Agency.

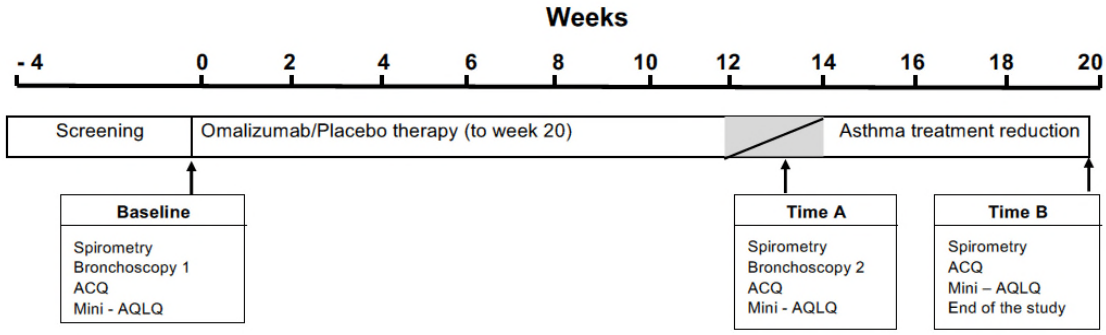


Figure 3.1: Clinical trial flow chart outlining interventions. **Baseline:** Time from screening visit to first bronchoscopy and commencement of omalizumab/placebo (Weeks -4 to 0). **Time A:** Time span during which the patients had a second bronchoscopy (Weeks 12 to 14) after which therapy was reduced. **Time B:** End of the trial 20 weeks from the first injection of omalizumab/placebo.

Clinical trial design, clinical interventions, immunohistochemical and immunofluorescence techniques are described in detail in chapter 2 (sections 2.2, 2.3, 2.4, 2.6, 2.7.1.1 and 2.7.2). The clinical interventions are also outlined in the trial flow chart (Figure 3.1).

3.4 Statistical methods for the clinical trial

3.4.1 Powering

As explained above, in this study I elected in the first instance to provide proof of concept in a double blind, placebo controlled study that omalizumab therapy can maintain or improve lung function in non-atopic asthmatics despite provocation of the disease in the short term by

supervised reduction of therapy. In a previous therapy reduction study (CQAE397A2202) performed by Novartis using patients of similar severity and taking similar therapy at baseline, the standard deviation of the differences in FEV_1 before and after reduction of inhaled corticosteroid was found to be 9.550. Using twenty subjects per group this would give a 90% chance of detecting a 10% difference in changes in FEV1, with an alpha of 0.1 using a two-sided test. Reduction in deterioration of the FEV1 following withdrawal of therapy over the 20 weeks of the study in the omalizumab, compared with the placebo treated group was thus set as the primary outcome measure. The immunohistochemical changes were unprecedented and so could not be formally powered. Nevertheless, the difference between the changes in the median numbers of bronchial mucosal IgE^+ cells in the active and placebo treated groups between baseline and time A was considered as a co-primary outcome since such a finding would furnish proof of principle that the omalizumab therapy as delivered had exerted tangible effects on binding of IgE to cells bearing its receptors within the target tissue.

3.4.2 Statistical analysis

Baseline characteristics and demographic data were summarised using descriptive statistics. Changes in numerical variables at the beginning and end of the study as well as differences in changes between the omalizumab and placebo treated groups were analysed by non-parametric statistics (Mann-Whitney U test). All tests were two sided and $p<0.05$ was accepted as significant. The statistical software package used was GraphPad Prism version 5.

3.5 Results

3.5.1 Patients

Of 30 patients screened, 18 were randomised (1:1) and 15 completed the study. The patients randomised to omalizumab or placebo therapy were well matched in terms of distributions of

age, sex, body mass index, serum total IgE concentrations, blood eosinophil counts, exhaled nitric oxide measurements, smoking history, lung function, asthma symptom scores and inhaled corticosteroid dosages (Table 3.3). There were 5 males and 3 females in the omalizumab group, and 3 males and 5 females in the placebo group. In the omalizumab *vs.* placebo treated groups, 7 *vs.* 8 patients were taking long-acting β2-agonist, 4 *vs.* 1 were taking oral leukotriene receptor antagonist and 2 *vs*. 2 were taking oral theophylline preparations. These medications were all stopped or substituted at Time A (Figure 3.1). 3 patients each in the omalizumab and placebo treatment groups had screening blood eosinophil counts > $0.4x10^9$/L, suggesting an equal distribution of so-called "eosinophilic" asthma between the groups, although this was not a selection criterion, nor was it hypothesised to influence the primary outcome measures.

3.5.2 Steroid reduction

One patient randomised to omalizumab and 3 randomised to placebo were taking oral prednisolone at dosages of 15 mg/day and 15, 10 and 5 mg/day respectively which were reduced to 7.5 mg/day and 7.5, 5 and 0 mg/day respectively according to the predetermined regimen as shown in tables 3.1 & 3.2.

Inhaled corticosteroid dosages were reduced in both groups to 400μg/day beclometasone equivalent (Symbicort 100/6 Turbohaler 2 puffs twice daily) between 12 and 16 weeks and further to 200 μg/day (Symbicort 100/6 Turbohaler 1 puff twice daily) between 16 and 20 weeks.

Table3.1: Protocol for the reduction of prednisolone dosage

Starting dose of Prednisolone (mg)	Time A (week 12-14)	Week 16	Time B (20 weeks)
20	15	10	10
17.5	12.5	7.5	7.5
15	10	7.5	7.5
12.5	7.5	5	5
10	7.5	5	5
7.5	5	2.5	2.5
5	2.5	0	0

Table 3.2: Prednisolone reduction in individual patients

Randomisation		Prednisolone dose (mg)		
		Baseline	Time A (week 12-14)	Time B (week 20)
Omalizumab		15	10	7.5
Placebo	i)	15	10	7.5
	ii)	10	7.5	5
	iii)	5	2.5	0

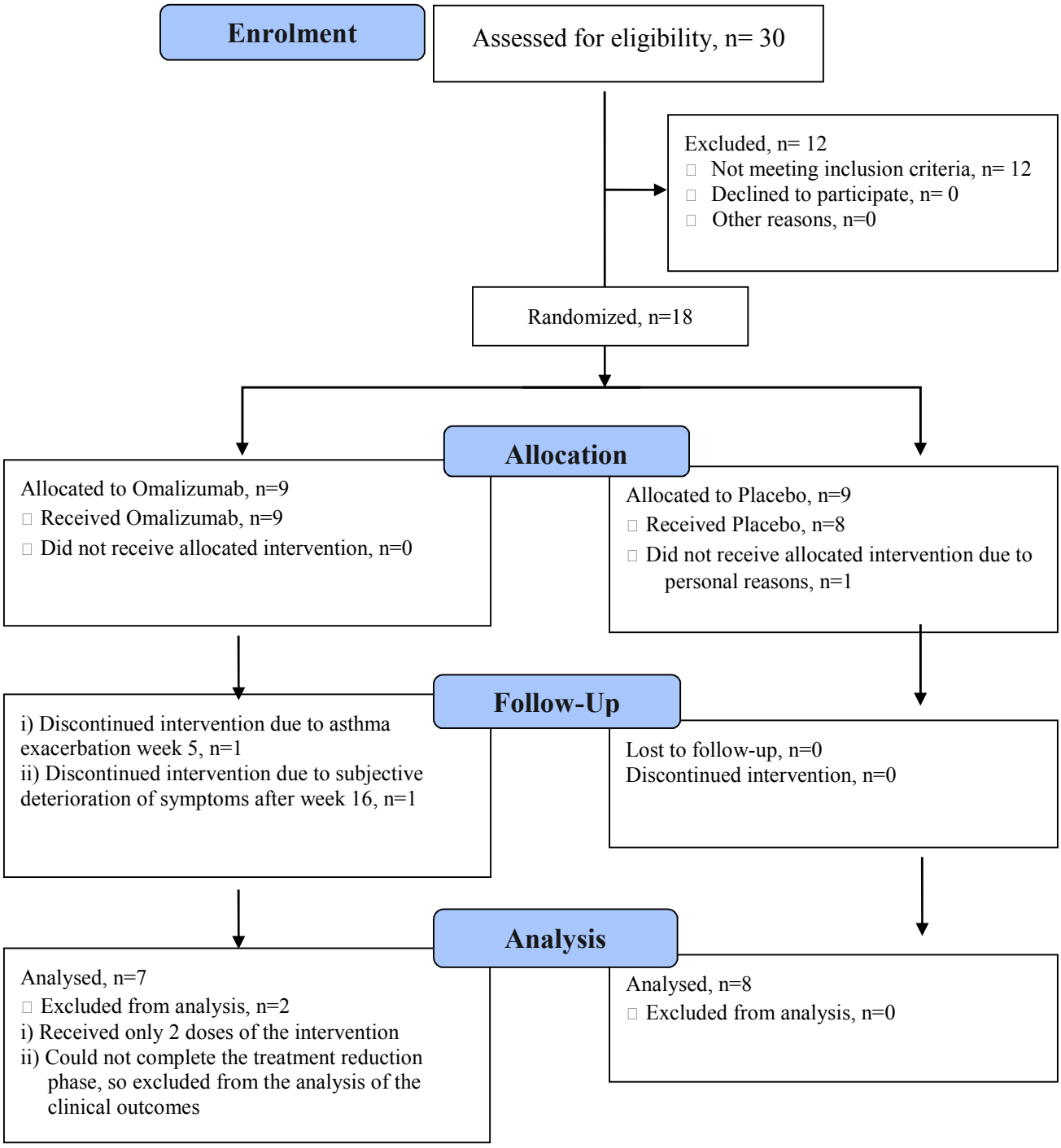
Characteristics & Outcomes	Randomisation	Baseline	12 -14 weeks (Time A) (Prior to reduction of therapy)	20 weeks (Time B)
Age (yr)	Omalizumab	47 (22, 66)		
	Placebo	53.50 (25, 59)		
Sex (M/F)	Omalizumab	5/3		
	Placebo	3/5		
Weight (Kg)	Omalizumab	84 (55, 133)		
	Placebo	87.50 (61, 112)		
Height (cm)	Omalizumab	172 (155, 178)		
	Placebo	166 (152, 177)		
Never smokers (n/8)	Omalizumab	3		
	Placebo	4		
Serum total IgE (IU/ml)	Omalizumab	75 (8, 264)		
	Placebo	49 (2, 284)		
Blood eosinophil count ($x10^9$/L)	Omalizumab	0.3 (0, 1)		
	Placebo	0.1 (0, 0.7)		
FEV_1 (L)	Omalizumab	2.44 (1.81, 3.43)	0.225 (-0.32, 1.24)	0.26 (0.03, 0.67) *
	Placebo	2.34 (1.16, 3.66)	0.005 (-0.30, 0.45)	-0.06 (-0.63, 0.39)
FEV1 % predicted	Omalizumab	75 (50, 98)	6 (-13, 36)	11 (1, 19) **
	Placebo	80 (47, 114)	-4 (-11, 14)	-2 (-23, 15)
PEFR (L)	Omalizumab	364 (233, 480)	10.50 (-3, 55)	17.50 (-34, 104)
	Placebo	308 (174, 455)	8.50 (-41, 92)	4 (-83, 111)
ACQ score	Omalizumab	2.28 (1.43, 3.43)	-0.50 (-1.85, 0.72)	-0.71 (-1.14, 0.14)
	Placebo	2.42 (0.71, 3.28)	-0.35 (-1.00, 0.57)	-0.28 (-1.71, 1.14)
AQLQ score	Omalizumab	4.33 (2.53, 5.27)	0.18 (-1.00, 2.26)	0.46 (-0.40, 2.06)
	Placebo	4.60 (3.73, 5.80)	0.37 (-1.34, 1.87)	0.67 (-1.60, 2.30)
ACD score	Omalizumab	1.86 (0.86, 2.84)	0 (-1.15, 0.45)	-0.28 (-1.21, 0.65)
	Placebo	1.97 (0.39, 2.80)	-0.26 (-1.45, 1.10)	-0.24 (-1.52, 2)
Exhaled NO (ppb)	Omalizumab	26.5 (18, 95)	36 (16, 80)	31 (12, 134)
	Placebo	21.5 (10, 42)	19 (5, 40)	20 (8, 142)
Inhaled corticosteroid dosage (BDP equivalent: µg/day)	Omalizumab	2000 (800, 4000)	Reduced to 400µg BDP equivalent/day	Reduced to 200 µg BDP equivalent/day
	Placebo	1800 (500, 2000)		

Table 3.3: Baseline demographics and clinical characteristics of non-atopic asthmatics randomised to omalizumab or placebo therapy and absolute changes from Baseline at Times A and B (see Figure 2.1). All variables are shown as the median and range. PEFR: Peak Expiratory Flow Rate, ACQ: Juniper Asthma Control Questionnaire, AQLQ: Juniper mini-Asthma Quality of Life Questionnaire, ACD: Asthma Control Diary. *p = 0.04, **p = 0.015 (Mann-Whitney U Test).

3.5.3 Adverse events and withdrawals (Figure 3.2: Consort diagram)

One patient from the placebo group withdrew after the screening visit for personal reasons. Two patients randomised to omalizumab therapy withdrew from the study prematurely, one following an asthma exacerbation (an expected adverse event) at week 5 and another who elected to withdraw after 16 weeks because of subjective deterioration of symptoms not confirmed by spirometry. There were no other adverse events.

Figure 3.2: Omalizumab Study CONSORT Flow Diagram



3.5.4 Primary outcome measure: FEV_1

Spirometry was performed at baseline and at Times A and B (Figure 3.1). Comparisons of changes in median absolute and % predicted FEV1 from baseline to Time A (12 - 14 weeks), and from Time A to B (12 - 20 weeks) did not show any statistically significant differences between the omalizumab and placebo groups. However, compared with baseline, in accordance with the planned primary outcome measure, changes in median absolute and % predicted FEV_1 by 20 weeks were positive in the omalizumab treated patients, despite substantial reduction of existing anti-asthma treatment, but negative in the placebo treated patients (median (SIQR) change 0.26 (0.07, 0.64) *vs*. -0.06 (-0.14, 0.27) litre, p=0.04; 11 (2, 18) *vs.* -2.0 (-11, 1.5) % predicted, p=0.015: see Figure 3.3 and Figure 3.4. (A comment from the statistician regarding the suitability and validity of this analysis is attached in Appendix 4).

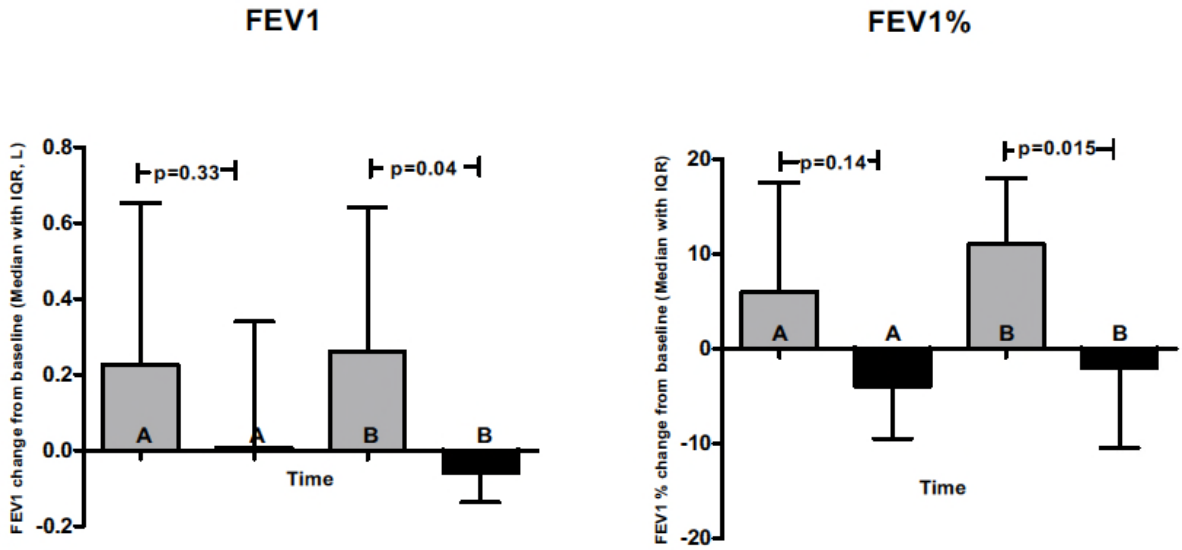


Figure 3.3: Comparison of effect of treatment with omalizumab and placebo on changes in absolute and % predicted FEV_1 between Baseline and Times A (12 - 14 weeks) and B (20 weeks) (see Figure 2.1 for definitions). Bars represent the median and interquartile range; Mann-Whitney U Test. Light shading: omalizumab treated patients; dark shading: placebo treated patients.

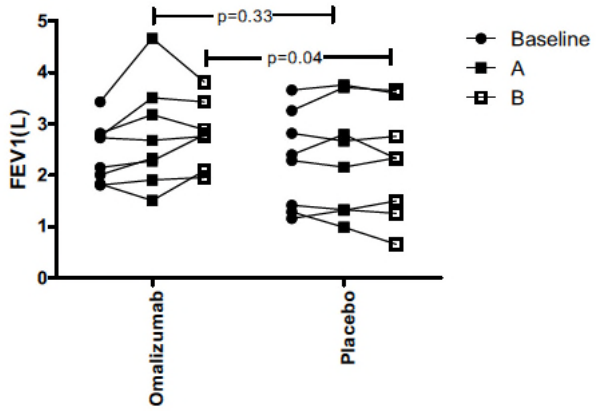


Figure 3.4: Comparison of effect of treatment with omalizumab and placebo on absolute FEV_1 of individual patients between Baseline and Times A (12-14 weeks) and B (20 weeks) (see Figure 3.1 for definitions). Light shading: omalizumab treated patients; dark shading: placebo treated patients.

3.5.5 Exploratory variables

As shown in Table 3.3, the median PEF and ACQ scores between baseline and Time B (20 weeks) improved by what is regarded as a clinically significant degree in the patients randomised to omalizumab therapy but not placebo, although the differences between the groups did not quite attain statistical significance in non-parametric testing. In contrast, ACD and mini-AQLQ scores improved to a similar extent in both groups despite reduction of conventional therapy.

3.5.6 Markers of airway inflammation

The median numbers of tryptase$^+$ mast cells, CD20$^+$ B cells, CD138$^+$ plasma cells, CD38$^+$ plasmablasts, total IgE$^+$ cells (most of these were likely mast cells), IgE$^+$/tryptase$^+$ mast cells, IgE$^+$/CD20$^+$ B cells, IgE$^+$/CD138$^+$ plasma cells, and BMK-13$^+$ eosinophils per unit area of the bronchial biopsy sections just prior to commencement of omalizumab and at Time A (after 12-14 weeks of omalizumab or placebo therapy with no alteration of existing medications, see Figure 3.1) are shown in Table 3.4, while selected changes are depicted graphically in Figure 3.5 and typical examples of the immunofluorescent and immunohistochemical techniques shown in Figure 3.6 and Figure 3.7 respectively. I observed a significant reduction in the median total number of IgE$^+$ cells ($p<0.001$), the co-primary outcome measure, in the bronchial mucosa in the patients treated with omalizumab but not placebo. No significant changes were observed in the median numbers of any of the other cells analysed following omalizumab or placebo therapy. Very few of the B cells and plasma cells showed detectable IgE immunoreactivity as expected (Table 3.4), so it was impracticable to evaluate changes. Conglomerates of CD38$^+$ plasmablasts and CD20+ B cells were detected in approximately 1 in every 10 sections of the mucosa (Figure 3.5F), but again these were insufficient in number to quantify possible changes.

Cell Type	Treatment Group	Absolute numbers/mm^2 at baseline	Difference between baseline and Time A (12-14 weeks prior to reduction of conventional therapy)
Tryptase$^+$ Mast Cell	Omalizumab	7.22 (0, 124.77)	10.88 (-61.64, 57.70)
	Placebo	29.92 (4.32, 75.64)	3.68 (-14.66, 13.08)
CD20$^+$ B Cell	Omalizumab	1.48 (0.00, 9.67)	0.00 (-8.25, 4.80)
	Placebo	2.10 (0.29, 9.35)	-0.15 (-1.32, 6.39)
CD138$^+$ Plasma Cell	Omalizumab	0.00 (0.00, 36.63)	0.00 (-4.42, 3.47)
	Placebo	1.68 (0.00, 6.80)	-0.24 (-1.93, 1.13)
CD38$^+$ Plasmablast	Omalizumab	3.91 (0.00, 14.57)	3.07 (-3.83, 69.72)
	Placebo	0.61(0.00, 39.39)	-0.21 (-28.39, 8.67)
IgE$^+$ Cell	Omalizumab	12.38 (1.22, 175.90)	-5.44 (-109.89, -1.30)**
	Placebo	16.45 (2.86, 60.92)	4.98 (-17.08, 17.81)
IgE$^+$ /Mast cell	Omalizumab	9.19 (0.00, 124.46)	-1.89 (-99.94, 33.31)
	Placebo	26.16 (4.32, 50.23)	7.88 (-17.58, 24.08)
IgE$^+$ /B cell	Omalizumab	0.00 (0.00, 0.28)	0.00 (0.00, 0.32)
	Placebo	0.00 (0.00, 0.00)	0.00 (0.00, 0.00)
IgE$^+$ /Plasma cell	Omalizumab	0.00 (0.00, 5.98)	0.00 (-5.41, 0.00)
	Placebo	0.00 (0.00, 0.46)	0.00 (-0.46, 0.00)
BMK-13$^+$ Eosinophils	Omalizumab	9.03 (2.92,17.20)	-0.76 (-14.40, 4.47)
	Placebo	4.26 (0.31, 21.36)	0.81 (-9.65, 7.57)

Table 3.4

Bronchial mucosal inflammatory cells: absolute counts (median, range) per mm^2 at baseline and change following treatment with omalizumab/placebo at Time A (see Figure 2.1).

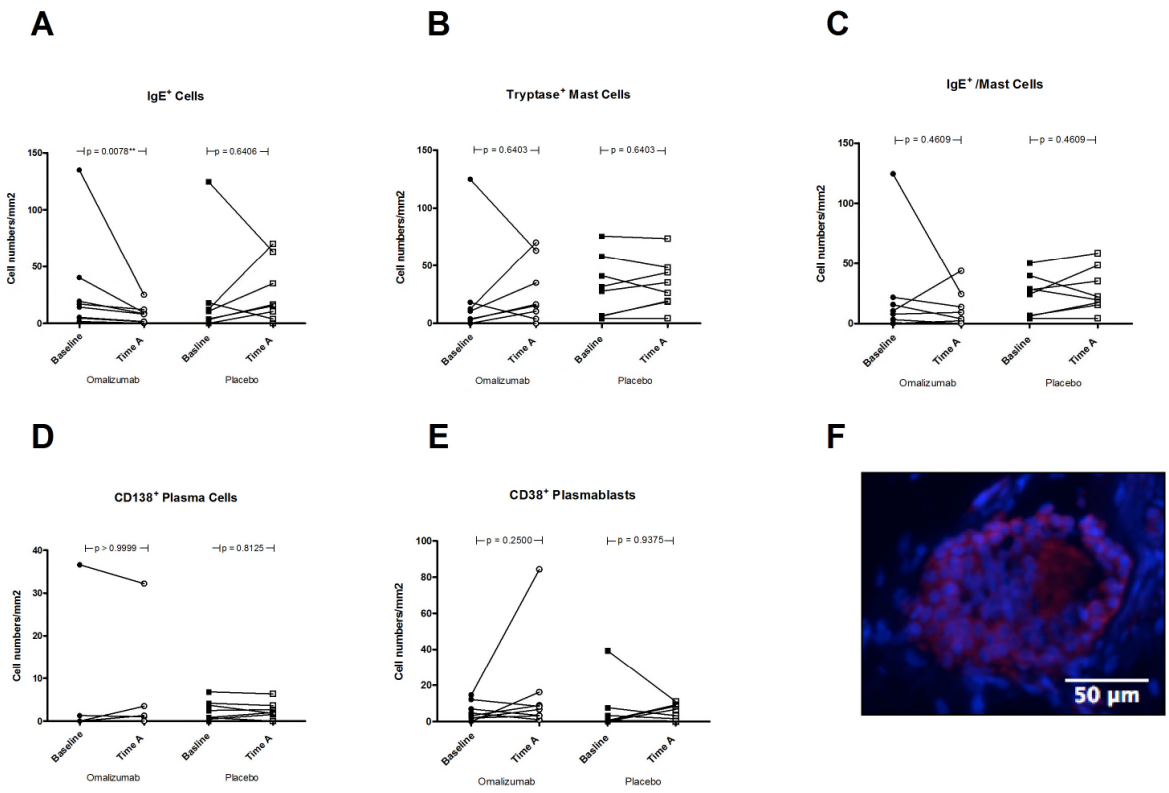


Figure 3.5

Effects of omalizumab and placebo treatment on numbers (cells/mm^2) of bronchial mucosal (A) total IgE$^+$ cells; (B) tryptase$^+$ mast cells; (C) IgE$^+$/tryptase$^+$ mast cells; (D) CD138$^+$ plasma cells; (E) CD38$^+$ plasmablasts. Median with interquartile range; Mann-Whitney U Test. (F) Immunofluorescence image of a typical conglomerate of plasmablasts in the bronchial mucosa stained with anti-CD38 (red) in a background of nucleoprotein (blue).

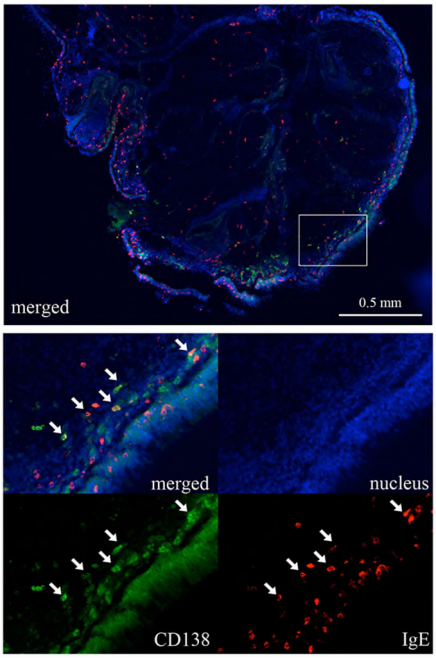
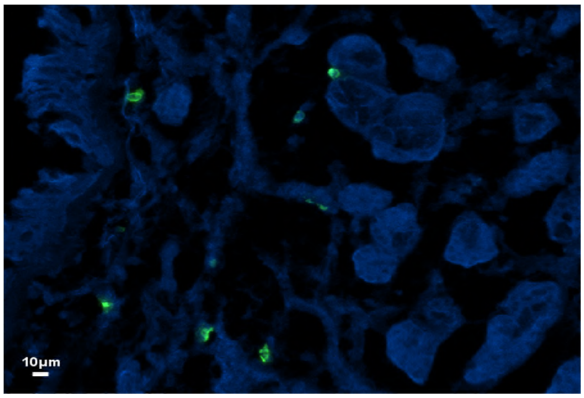
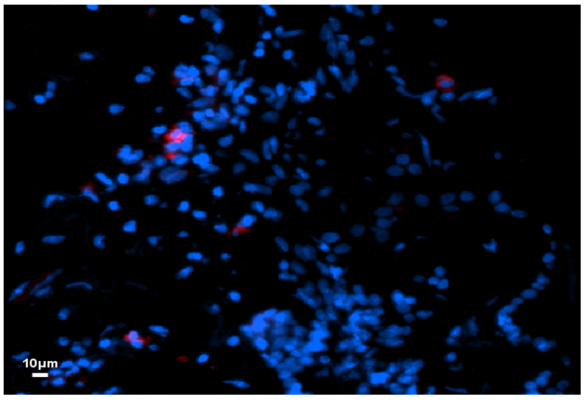


Figure 3.6: Immunofluorescence image of bronchial biopsy from a non-atopic asthmatic patient stained for plasma cells (CD138; green), IgE (red) and nucleus (blue). Top: View of whole biopsy, showing area of magnification. Bottom: Magnified view, with a merged image and individual stains. Arrows indicate cells with dual staining (plasma cells expressing IgE).

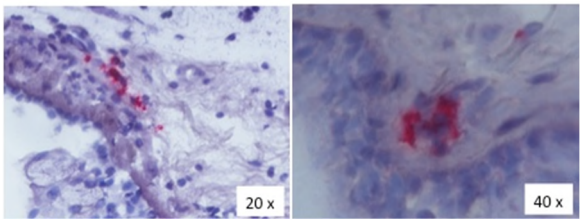
Figure 3.7: Immunofluorescence & immunohistochemistry images of bronchial biopsies from non-atopic asthma patients



Tryptase + cells (green)



CD 138+ Plasma cells (red)



BMK 13+ eosinophils

3.6 Discussion

This section of my PhD thesis was conceived to challenge, in the light of current knowledge, the tacit assumption that omalizumab therapy is of no potential clinical benefit in conventionally defined, non-atopic asthmatics. In place of a large, long-term, conventional clinical trial in the first instance, which would have been difficult to fund due to the paucity of supportive evidence, I wished to provide proof of concept that omalizumab therapy of these patients exerts a favourable effect on lung function, as assessed by changes in the FEV_1, and reduces IgE expression and IgE sensitisation of target cells within the bronchial mucosa (co-primary outcome measures) following a period of therapy with omalizumab followed later by withdrawal of existing, conventional therapy. I first obtained bronchial biopsies before and after 12-14 weeks of treatment of the non-atopic, asthmatic patients with omalizumab or placebo while maintaining existing therapy in order to address the hypothesis that omalizumab compared with placebo, under conditions where conventional asthma therapy is maintained, reduces local IgE binding to inflammatory cells within the bronchial mucosa, the co-primary outcome measure. To obtain proof of concept that omalizumab can improve lung function, I then destabilised the patients by staged reduction of their therapy according to a protocol previously validated by Novartis and powered to detect changes in FEV_1, the second, co-primary outcome measure. Although I was unable, despite my highest and most strenuous endeavour, to recruit as many subjects as demanded by the power calculation because recruitment to this complex and demanding study proved challenging (see further comments below), I did nevertheless did observe a significant improvement in the median FEV_1 when compared with baseline in those patients who received omalizumab, but not placebo, over the 20 week period of the study, incorporating the therapy reduction phase on which the study was powered and also incidentally comparable to the time frame used to gauge responsiveness clinically. I also explored changes in quality of life and asthma symptom scores and, although

the study was not powered to examine these it revealed a modest improvement in the ACQ, but not the mini-AQLQ score. It is arguable that the ACQ score is more reflective of short-term asthma stability than mini-AQLQ, which may improve in response to increased surveillance in the context of a clinical trial. These data are congruent with a previous placebo-controlled proof of concept clinical trial by Garcia and colleagues examining the clinical effects of omalizumab in non-atopic asthmatics[207], who were also able to demonstrate improvement of FEV_1 in their patients and a trend towards improvement in global evaluation of clinical effectiveness and asthma exacerbation rate and another observational study using data from Spanish multicentre registry[208].

Two patients randomised to omalizumab therapy withdrew, the first following an exacerbation just after commencing omalizumab and prior to any change in therapy. The second patient elected to withdraw during the therapy reduction phase despite lack of any objective evidence of deterioration (please refer the consort diagram, Figure 3.2). The data from this patient's bronchial biopsies, but none of the clinical data were included in the final analysis. Although in larger studies withdrawal rates may reflect the efficacy of a novel therapy, this is clearly not applicable in the present study. One patient assigned to omalizumab and three to placebo were taking oral corticosteroids. Since we hypothesised that omalizumab reduces IgE expression in the target organ of non-atopic asthmatics whose existing therapy is maintained, and that omalizumab therapy preserves/improves FEV_1 in the face of significant therapy reduction, it is unlikely that my conclusions have been influenced by this.

Having demonstrated that IgE is increased in the bronchial mucosa of non-atopic asthmatics[188], I here additionally show that omalizumab therapy substantially reduces the numbers of cells (likely principally mast cells) exhibiting associated IgE, consistent with my hypothesis (and also incidentally clearly suggesting that omalizumab penetrates the bronchial

mucosa when administered therapeutically to these patients). This presumably reflects sequestering by omalizumab of free IgE within the bronchial mucosa, resulting in down regulation of its receptors on local target cells [128,209,210]. Similarly, in a study of mild atopic asthmatics[185], treatment with omalizumab was associated with a reduction in the numbers of bronchial mucosal FcεRI$^+$, IgE$^+$ cells, eosinophils, T lymphocytes and B lymphocytes.

Returning to the question of why inhibition of binding of IgE to its receptors, which is the only established, direct anti-inflammatory action of omalizumab may benefit asthma regardless of conventional atopic status, it is worth examining the data from the INNOVATE study post-hoc analysis which suggested that subgroups of the severe allergic asthmatics participating in this study with relatively high baseline eosinophil counts derive the greatest clinical benefit from omalizumab[211]. Although such post-hoc conclusions should properly be regarded with caution, this observation is consistent with data discussed previously in the introductory chapter suggesting that one of the principal actions of omalizumab may be to restore asthma stability by restoring innate, anti-viral immunity. This may partly account for the INNOVATE post-hoc analysis findings, since asthma patients who are relatively unstable and in and out of hospital despite being on high step anti-asthma therapy are often patients with relatively high blood eosinophil counts and frequent viral exacerbations of disease. Of course my own study was neither intended nor powered to examine variation in responsiveness to omalizumab according to blood eosinophil counts.

Whatever the precise anti-inflammatory actions of omalizumab, it seems clear that its clinical effectiveness varies in individual patients in a manner not predicted by their baseline, circulating IgE concentrations[212], suggesting that responsiveness does not simply reflect overall IgE production (at least in the circulation). In addition, Chanez and colleagues[213] showed that, while in a group of severe atopic asthmatics omalizumab therapy reduced

expression of FcεRI on blood basophils and plasmacytoid dendritic cells (a finding also confirmed in the study by Garcia and colleagues[214]), there was no correlation with clinical responsiveness.

To further explore possible functional consequences of omalizumab therapy I looked for changes in inflammatory cell numbers in the bronchial mucosa (such changes cannot prove cause and effect but may provide mechanistic clues). In the event I observed none. One caveat is the issue of cell clusters. It was with great interest that I noticed clusters of $CD38^+$ plasmablasts and CD20+ B cells in approximately 1 in 10 sections of the bronchial mucosa, consistent with the previous observations in the literature that these cells switch locally to IgE synthesis [53]: this would likely be accompanied by multiple cellular divisions[215]. It is technically challenging to enumerate changes in numbers of cells distributed as discrete clusters. Even assuming, however, that omalizumab does also inhibit local B cell differentiation and IgE synthesis in the bronchial mucosa, consistent with its known effects on IgE expressing B cells and antigen-presenting cells [216,217], it is again not yet absolutely clear if and why this may be important in asthma.

3.7 Issues and shortcomings of the study

The original objective when designing this clinical trial was to recruit 40 participants in order to be certain of having the statistical power to detect a difference in changes in FEV_1 between the omalizumab and placebo treated groups. At one stage when thinking about the design of the original trial protocol is was mooted that asthma exacerbation rate, defined as a need for rescue oral prednisolone treatment for deteriorating symptoms and/or lung function in the therapy reduction phase of the study should also be examined as an outcome measure, but it quickly became clear that, with such a short trial this was out of the question because of the paucity of such events (in fact only one of the patients suffered a true disease exacerbation

during the trial and this was not during the therapy reduction phase). It was also planned (and executed) to monitor, speculatively, a full range of additional, conventional clinical outcome measures (symptom scores, QoL scores and FeNO). Powering is designed principally to eliminate the possibility of Type 2 error, which refers to the situation when a significant difference between two sets of variables is obscured by the inherent variation of the variables being measured. Fortunately, in this study a statistically significant difference between the groups was indeed observed in the primary outcome measure variable (FEV_1) despite under recruitment (<50% of the intended sample size), which by definition obviates Type 2 error. I should add that I did everything in my power to attain this recruitment target. The main difficulty encountered during recruitment was identifying sufficient numbers of eligible, moderate/severe non-atopic asthmatic patients satisfying all of the inclusion & exclusion criteria. Many non-atopic asthmatics failed screening based on their failure to meet the (*ad hoc* but conventional) spirometric criteria used to define asthma, their smoking status (although I felt obliged to accept ex-smokers who met the inclusion criteria) and the time interval from a previous exacerbation. Many otherwise likely eligible candidates were unwilling to consider participation because of the time commitment involved, the necessity for time off work and the prospect of undergoing two successive bronchoscopies with bronchial biopsy. Finally, I believe it is fair to state that the assistance I anticipated with recruitment of suitable patients from the other two centres was far from as extensive as I had hoped (in fact I personally recruited all but two of the patients in this study).

Chapter 4: Total and allergen-specific IgE in the bronchial mucosa of atopic and non-atopic asthmatics and non-atopic controls

4.1 Introduction

IgE binds to its high affinity receptor FcεRI on mast cells in the target organs of allergy in surveillance for antigen. The cognate interaction of a multivalent antigen cross-links the receptors to induce mast cell activation. Activated mast cells release mediators of the immediate reaction to the antigen (allergen) and initiate an inflammatory cascade: allergic inflammation. This in turn may result in exacerbation (some speculate causation) of asthma, although in many patients with peripheral, allergen-specific IgE allergen exposure has no obvious clinical sequelae. A similar clinical syndrome is manifest in "non-atopic" asthma, in the apparent absence of provoking allergens, as typically deduced from negative skin prick tests with a panel of common allergens or the absence of detectable allergen-specific IgE in serum. Thus, the propensity for allergen or indeed IgE to provoke non-atopic asthma is unclear. We have shown, however in previous studies[119,123,202] that the entire molecular machinery to support local B cell IgE switching is in place in the bronchial mucosa of non-atopic, as well as atopic asthmatics and that switching does indeed occur at this site[126,218]. Finally, local expression of FcεRI on inflammatory cells is elevated to an equivalent degree in the bronchial mucosa of non-atopic, as well as atopic asthmatics[39], which is also consistent with local IgE concentrations in excess of those in the serum of these patients since IgE up regulates expression of its own high-affinity receptor[219]. Furthermore, epidemiologically, elevated IgE production is a significant risk factor for asthma regardless of its specificity[125,220].

One possible explanation for this paradox is that aeroallergens may provoke a local, specific IgE response in non-atopic asthmatics in the bronchial mucosa but that this IgE is captured by local mast cells in the tissue and does not reach peripheral mast cells in the skin or the circulation. There is some precedent for this in non-atopic rhinitis[90] .

To begin to solve this paradox in the present study, I first sought to determine whether some of the patients classified as non-atopic asthmatics had IgE antibodies against known allergens, but that these IgEs were confined to the bronchial mucosa. This was an obvious possibility given preliminary similar findings in non-atopic rhinitis[90] and the continuity of the respiratory tract mucosa from the nose to the lung.

Accordingly, I compared the concentrations and specificities of the IgE in the blood and bronchial mucosa of atopic and non-atopic asthmatics and non-atopic controls. I hypothesised that local IgE synthesis in the bronchial mucosa of both atopic and non-atopic asthmatics results in greater local total IgE concentrations than in the serum and, in the case of non-atopic asthmatics, production of allergen-specific IgE which is sequestered in the mucosa and therefore not detectable in the periphery. I tested the specificities of IgEs in both compartments, not only to the panel of allergens originally used in the skin prick tests, but also to a panel of over 100 known allergen components, to investigate the further possibility that the IgEs may be specific for uncommon allergens.

4.2 Materials and methods

A total of 10 each of atopic & non-atopic asthmatics and non-atopic non-asthmatic controls were recruited and bronchial mucosal biopsies and blood samples collected as described in Chapter 2: sections 2.1, 2.2, 2.3, 2.5 and 2.6. Further sample processing, protein extraction methods and IgE assay (ImmunoCAP and ISAC) are described in detail in Chapter 2: sections 2.7.1.1, 2.7.1.3 and 2.7.3

4.3 Statistical analysis

Statistical analysis was performed using Kruskal-Wallis ANOVA & Dunn's multiple comparisons test. The statistical software used was GraphPad Prism, version 5.

4.4 Results

Relevant clinical and demographic details of the subjects are summarised in Tables 4.1 and 4.2. The median ages of the atopic and non-atopic asthmatics were not statistically different although significantly higher in both groups than those of the controls. The median FEV_1 of the non-atopic asthmatics was significantly lower than that of the atopic asthmatics and the controls, while the median FEV_1 of the milder atopic asthmatics and controls did not significantly differ. The non-atopic asthmatics were taking a significantly elevated median daily dosage of inhaled corticosteroid compared with the atopic asthmatics, in accordance with their more severe disease.

4.5 Total IgE in bronchial biopsies and serum

IgE was detectable in the serum and bronchial mucosa of all of the atopic and non-atopic asthmatics and non-atopic controls (Figure 4.1). The median serum total IgE concentration was significantly elevated in the atopic ($p = 0.001$), but not the non-atopic ($p = 0.32$) asthmatics as compared with the non-atopic controls (Figure 4.2). In contrast, the median total IgE concentration in the bronchial mucosal homogenates was significantly elevated in both the non-atopic ($p = 0.003$) and the atopic asthmatics ($p = 0.038$) compared with the controls. These differences remained statistically significant even after allowing for variability of IgE concentrations between different mucosal sites as described below. Comparing the atopic and

non-atopic asthmatics, there was no significant difference in the median total IgE concentrations either in the mucosa ($p > 0.99$) or in the serum ($p = 0.14$).

Subject group	Non-atopic controls	Non-atopic asthmatics	Atopic asthmatics
Age (yr)	**23 (21-36)**	**54 (24 - 70) ***	**30.50 (24-66) ***
Sex (F/M)	**3/7**	**4/6**	**4/6**
FEV1 (% predicted)	**104.5 (94-128)**	**67.5 (40-101) ****	**95 (64-130) #**
Blood eosinophil count (x 10^9/L)	**0.1 (0-0.4)**	**0.25 (0-0.7)**	**0.35 (0.1-1.1) ***
Inhaled corticosteroid dosage (µg/day BDP equivalent)		**1800 (640-4000)**	**150 (0-1600) ##**

Table 4.1: Demographics & lung function of study subjects and corticosteroid therapy of asthmatics. Age, FEV_1 % predicted, blood eosinophil count at recruitment and inhaled corticosteroid dosages are expressed as the median (range). 3 non-atopic asthmatics were taking regular oral prednisolone in addition to inhaled corticosteroids. (F: Female; M: Male; BDP: Beclometasone dipropionate). *$p \leq 0.01$; **$p \leq 0.005$ vs. controls; #$p=0.02$; ##$p=0.002$ vs. non-atopic asthmatics (Kruskal-Wallis ANOVA & Dunn's multiple comparisons test).

No.	Age	Sex	Positive sensitisation status
1	24	F	Cat, Dog
2	29	M	Grass, Tree, Cat, Dog, HDM, Mould
3	66	F	Grass, Cat, Dog, HDM, Mould
4	32	F	Grass, Cat, HDM, Mould
5	28	M	Grass, Tree, Cat, Dog, HDM, Mould
6	54	M	Tree, Cat, Dog, HDM, Mould
7	24	F	HDM
8	26	M	Grass, Cat, HDM
9	47	M	Mould
10	35	M	Tree, Grass, HDM, Mould, Cat, Dog

Table 4.2: "Conventional" allergen sensitisation of atopic asthmatics. (HDM: house dust mite).

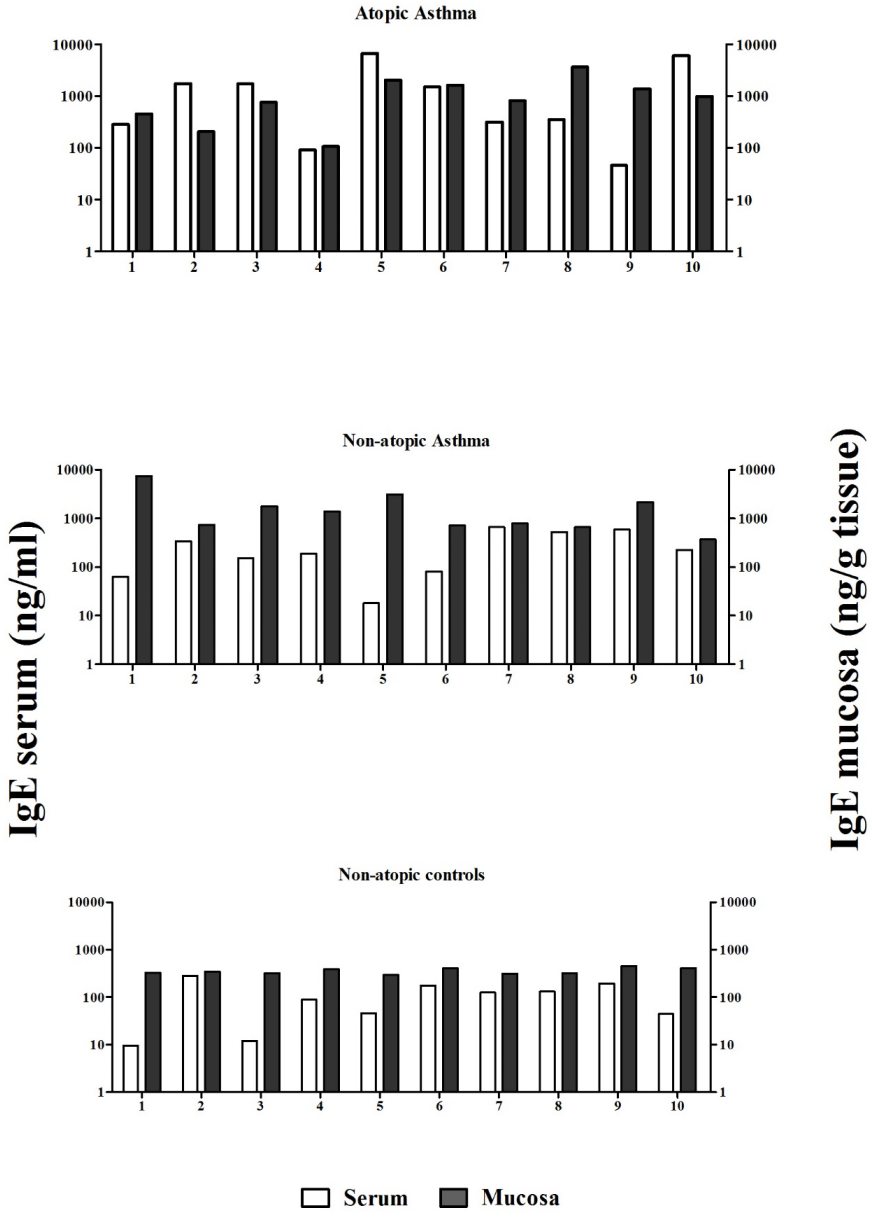


Figure 4.1

Total IgE concentrations in the serum (left Y axes, open bars) and the bronchial mucosa (right Y axes, shaded bars) of atopic asthmatics (top), non-atopic asthmatics (middle) and non-atopic controls (bottom).

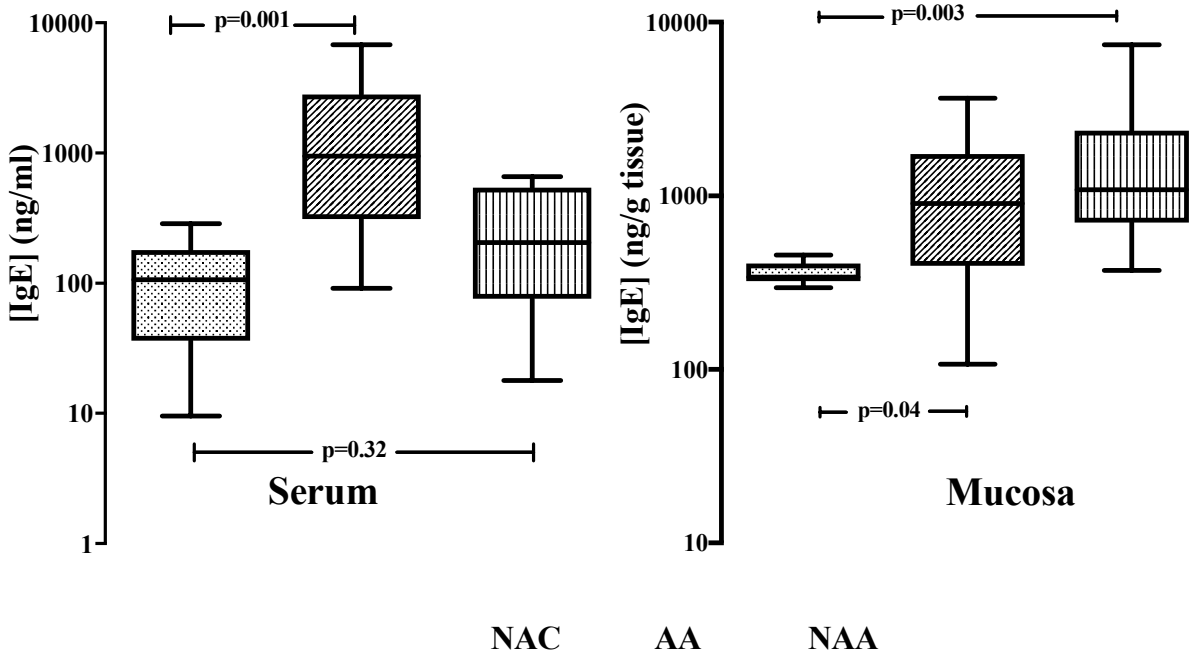


Figure 4.2

Box and whisker plots summarising total IgE concentrations in the serum (left) and bronchial mucosa (right) of the non-atopic controls (NAC), atopic asthmatics (AA) and non-atopic asthmatics (NAA). Kruskal-Wallis ANOVA & Dunn's multiple comparisons test.

4.6 Component-specific IgE

Concentrations of IgE specific for a range of common and some uncommon allergen components were measured using ImmunoCAP ISAC microarray in the three subject groups. Allergen component-specific IgE species were detectable in the sera and biopsy homogenates of the atopic asthmatics. The relative concentrations of a minority of these components, expressed as percentages of the total IgE, were higher in the mucosa than in the serum. Figures 4.3, 4.4 & 4.5 and tables 4.3, 4.4 & 4.5 show only positive data obtained from the standard ISAC assay. In some of the atopic subjects sensitised to particular allergens by conventional criteria (SPT or PhadiaCAP), IgE against none of the corresponding allergen components in the standard ISAC chip was detectable in the serum or the bronchial mucosa (subjects sensitised to - grass: 3, 4 and 8; tree: 2; moulds: 3, 4, 5 and 6; cat: 4; dog: 3). Component specific IgE concentrations did not exceed the threshold of detection of the ISAC assay in the sera and mucosa of the non-atopic asthmatics or controls, in agreement with their negative skin prick and PhadiaCAP test results.

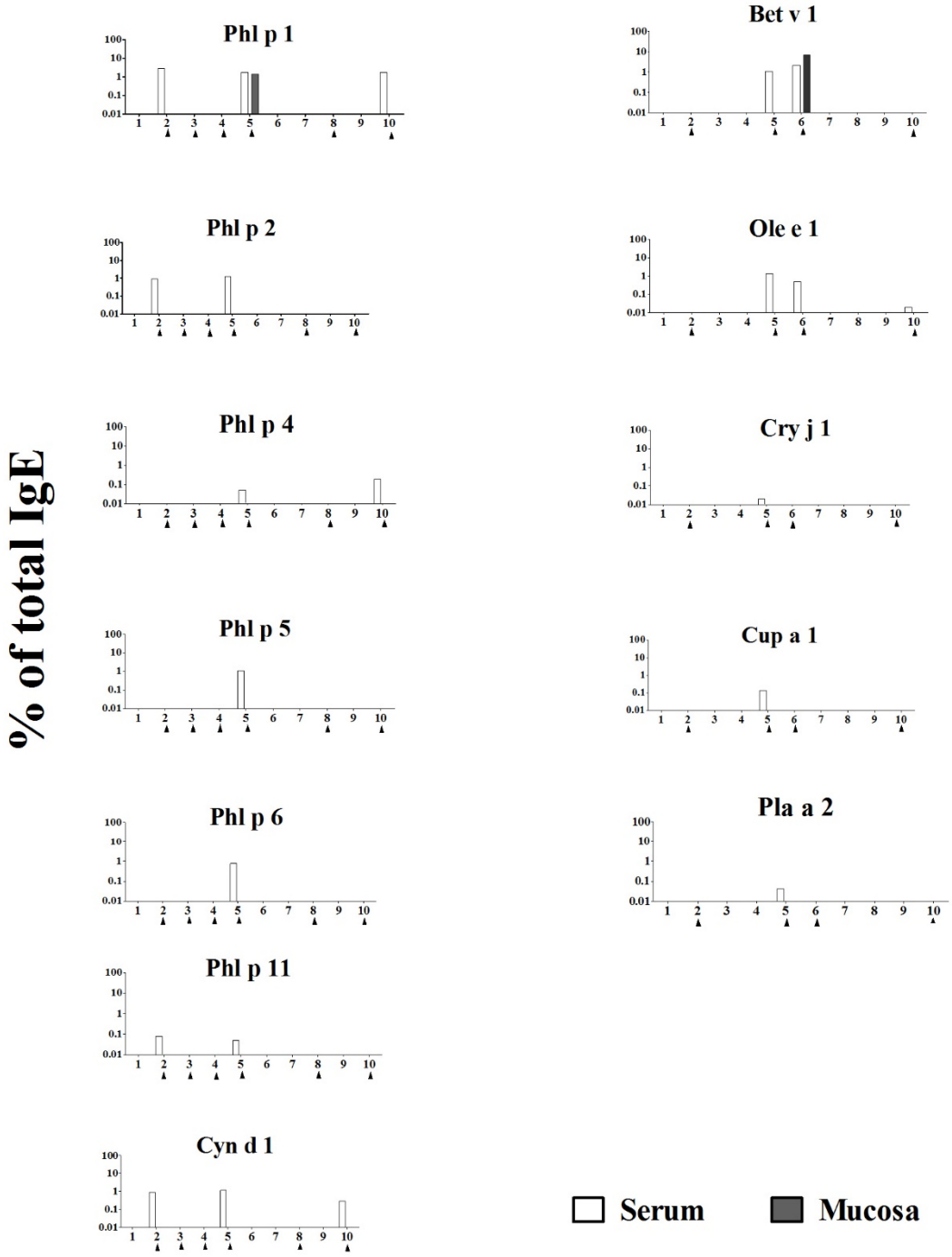


Figure 4.3

Concentrations of IgE specific for grass (Phl p 1, Phl p 2, Phl p 4, Phl p 5, Phl p 6, Phl p 11 and Cyn d 1*)* and tree (Bet v 1, Ole e 1, Cry j 1, Cup a 1 and Pla a 2) allergen components are expressed as percentages of the total IgE in the sera (open bars) and the bronchial mucosa (filled bars) of atopic asthmatics. Subjects sensitised to the corresponding allergens by SPT/ImmunoCAP are indicated by arrowheads.

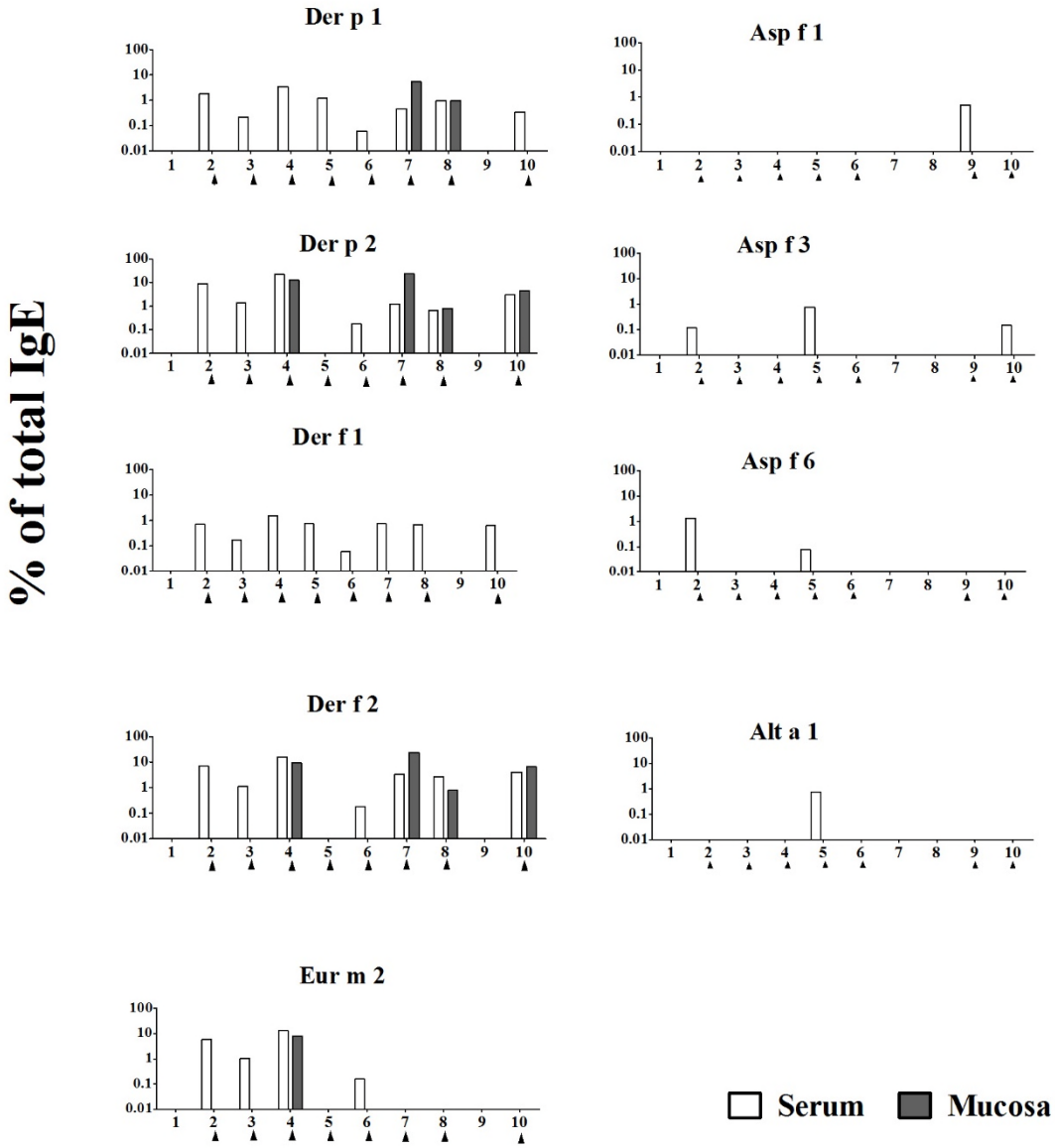


Figure 4.4

Concentrations of IgE specific for HDM (Der p 1, Der p 2, Der f 1, Der f 2 and Eur m 2) and mould (Asp f 1, Asp f 3, Asp f 6 and Alt a 1) allergen components are expressed as percentages of the total IgE in the sera (open bars) and the bronchial mucosa (filled bars) of atopic asthmatics. Subjects sensitised to the corresponding allergens by SPT/ImmunoCAP are indicated by arrowheads.

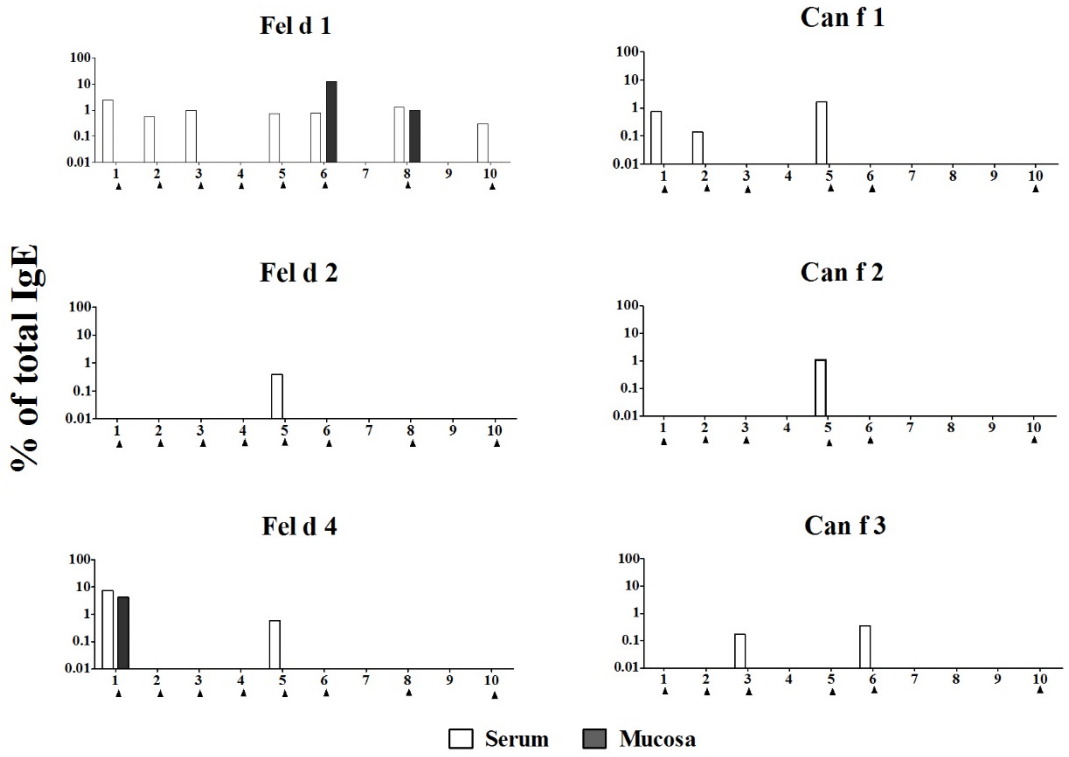


Figure 4.5

Concentrations of IgE specific for cat (Fel d 1, Fel d 2 and Fel d 4*),* and dog (Can f 1, Can f 2 and Can f 3) allergen components are expressed as percentages of the total IgE in the sera (open bars) and the bronchial mucosa (filled bars) of atopic asthmatics. Subjects sensitised to the corresponding allergens by SPT/ImmunoCAP are indicated by arrowheads.

Table 4.3: Allergen component (Grass and Tree) specific IgE concentrations from the serum (ng/ml) and bronchial mucosa (ng/g) of atopic asthmatics. Bronchial mucosal biopsy weights from each individual are also listed.

Subject no.	Tissue compartment	**Tissue weight in mg**	**Grass**								**Tree**					
			Phl p 1		**Phl p 2**	**Phl p 4**	**Phl p 5**	**Phl p 6**	**Phl p 11**	**Cyn d 1**	**Bet v 1**		**Cry j 1**	**Cup a 1**	**Ole e 1**	**Pla a 2**
			ng/ml	ng/g	ng/ml	ng/ml	ng/ml	ng/ml	ng/ml	ng/ml	ng/ml	ng/g	ng/ml	ng/ml	ng/ml	ng/ml
1	Mucosa	7.50														
	Serum															
2	Mucosa	15.00														
	Serum		50.82		16.70				1.45	15.97						
3	Mucosa	4.70														
	Serum															
4	Mucosa	7.00														
	Serum															
5	Mucosa	6.00		29.04												
	Serum		121.00		84.70	3.63	70.18	53.24	3.63	79.86	75.02		1.21	9.44	94.38	2.90
6	Mucosa	6.50										107.22				
	Serum										31.46				7.74	
7	Mucosa	12.00														
	Serum															
8	Mucosa	3.60														
	Serum															
9	Mucosa	1.84														
	Serum															
10	Mucosa	15.40														
	Serum		106.48			11.37				15.25					1.45	

Table 4.4: Allergen component (HDM) specific IgE concentrations from the serum (ng/ml) and bronchial mucosa (ng/g) of atopic asthmatics.

Subject no.	Tissue compartment	HDM											
		Der p 1		Der p 2		Der f 1	Der f 2		Eur m 2		Lep d 2		Blo t 5
		ng/ml	ng/g	ng/ml	ng/g	ng/ml	ng/ml	ng/g	ng/ml	ng/g	ng/ml	ng/g	ng/ml
1	Mucosa												
	Serum												
2	Mucosa												
	Serum	31.46		157.30		12.83	125.84		104.06				
3	Mucosa												
	Serum	3.87		24.20		2.90	19.84		18.15				
4	Mucosa				13.83			10.37		8.64			
	Serum	3.15		20.33		1.45	14.52		12.58				
5	Mucosa												
	Serum	82.28				50.82							
6	Mucosa												
	Serum	0.97		3.15		0.97	2.66		2.42				
7	Mucosa		43.56		188.76			196.02					
	Serum	1.45		3.87		2.42	10.65						
8	Mucosa		36.30		29.04			29.04			0.97	29.04	
	Serum	3.39		2.42		2.42	9.68				3.87		
9	Mucosa												
	Serum												
10	Mucosa				43.56			65.34					
	Serum	20.57		193.60		38.72	242.00						29.04

Table 4.5: Allergen component (Cat, Dog and Mould) specific IgE concentrations from the serum (ng/ml) and bronchial mucosa (ng/g) of atopic asthmatics

Subject no.	Tissue compartment	Cat					Dog			Mould		
		Fel d 1		Fel d 2	Fel d 4		Can f 1	Can f 2	Can f 3	Asp f 1	Asp f 3	Asp f 6
		ng/ml	ng/g	ng/ml	ng/ml	ng/g	ng/ml	ng/ml	ng/ml	ng/ml	ng/ml	ng/ml
1	Mucosa					19.36						
	Serum	6.78			21.30		2.18					
2	Mucosa											
	Serum	9.92					2.42				2.18	23.72
3	Mucosa											
	Serum	17.18							2.90			
4	Mucosa											
	Serum											
5	Mucosa											
	Serum	50.82		26.62	38.72		111.32	77.44	24.20		50.82	5.08
6	Mucosa		207.75									
	Serum	11.86										
7	Mucosa											
	Serum											
8	Mucosa		36.30									
	Serum	4.60										
9	Mucosa											
	Serum									1.94		
10	Mucosa											
	Serum	17.91									9.20	

4.7 IgE recovery

A maximum of approximately 50% of the total IgE originally present in the mucosal biopsies was lost during extraction. The most significant loss (approximately 40%) was following concentration of the homogenates using the Vivaspin2 spin columns, and then during detergent (CHAPS) removal (approximately 10%). This latter step was deemed necessary since CHAPS inhibited specific IgE detection by ISAC.

4.8 Variability between biopsies

IgE concentrations between multiple (n=10) biopsies from a single atopic asthmatic subject varied by a maximum of approximately two fold (median 1180 ng/g tissue, range 837 - 2531, interquartile range 783) (Table: 4.6).

	Component specific IgEs										
	Grass			Tree	Cat	Mould	HDM				
	Cyn d 1	Phl p 1	Phl p 4	Ole e 1	Fel d 1	Asp f 3	Blo t 5	Der f 1	Der f 2	Der p 1	Der p 2
Biopsy no.											
1									1.1		1.2
2											
3									0.9		0.6
4											
5											
6		0.7			0.6			0.8	3		3.3
7									1		0.9
8											
9											
Serum	6.3	44	4.7	0.6	7.4	3.8	12	16	>100	8.5	80

Table 4.6: Component specific IgEs detected by ISAC technique from serum and 9 different bronchial mucosal sites of an atopic asthmatic subject. Results are expressed in ISAC standardised units (ISU)

4.9 Discussion

So far as I am aware this is the first study in which total and allergen-specific IgE concentrations in the bronchial mucosa of asthmatics, both atopic and non-atopic, have been measured successfully. Arguably this is a more meaningful site at which to measure it since presumably mucosal IgE at least initiates IgE-mediated reactions to allergens.

As expected, I observed a significantly elevated median total serum IgE concentration in the atopic asthmatics as compared with the non-atopic controls. In the non-atopic asthmatics, the median serum IgE concentration was also elevated, albeit not significantly. This is consistent with previous studies showing elevated serum total IgE concentrations compared with controls in non-atopic asthmatics[113] and epidemiological studies[125,220] demonstrating that elevated serum IgE concentration is a risk factor for asthma regardless of its specificity. The measurements of total IgE in the bronchial mucosa of non-atopic asthmatics are entirely novel and demonstrate a median total IgE concentration in this compartment at least comparable with that in atopic asthmatics and clearly elevated compared with control subjects. These observations support the hypothesis that local IgE synthesis or at least sequestration is ongoing in this compartment in both atopic and non-atopic asthmatics.

When expressing total IgE "concentrations" in the bronchial mucosa I chose to express these per unit weight of wet tissue, reasoning that water with a density of approximately 1 g/cm^3 accounts for the bulk of the intracellular and extracellular content of the biopsies. Direct comparison of IgE "concentrations" in two structurally and physiologically distinct compartments such as the peripheral blood and the bronchial mucosa within individuals may not of course be appropriate and I did not attempt this.

I was able to apply the ISAC technique successfully to measure allergen-specific IgE in the bronchial biopsies and serum from the same patients. The aim was to understand the range of specificities of the IgE in the bronchial mucosa and serum of both atopic and non-atopic asthmatics, with the rationale that if allergen-specific IgE is produced in the bronchial mucosa and then partially or completely sequestered in the tissue, then the total concentrations and the range of such specificities might be higher and broader in the mucosa than in the serum. What was found, however was that for a range of common local aeroallergens (house dust mite, grass, tree, moulds, cat epithelium and dog epithelium), while most of the atopic asthmatics sensitised by conventional skin prick testing and/or PhadiaCAP to these allergens had detectable concentrations of at least some components of these allergens in the serum, IgE against a much smaller range of the components was detectable in the bronchial mucosa. Furthermore, and notwithstanding reservations about direct comparisons of the "concentrations" of IgE in these two distinct compartments, there was little evidence that local mucosal IgE concentrations were markedly elevated in comparison with concentrations in the serum. The fact that some atopic subjects sensitised by conventional skin prick test and/or Phadia CAP to grass pollen, tree pollen, moulds, cat and dog did not have detectable IgE in the serum or bronchial mucosa to any of the ISAC chip components suggests that the repertoire of IgE against allergen components detected by the ISAC chip is narrower than that produced by patients, or that the threshold of sensitivity of the assay for IgE against different allergen components is variable or both. It is also possible, however, that the range of specificities in a single bronchial biopsy may be unrepresentative of the full IgE repertoire in the bronchial mucosa, even though site to site differences in the total IgE concentrations were minimal. Regional differences may reflect the clonal proliferation of allergen-specific B cells at different sites. Another consideration is that local IgE

production against seasonal allergens might conceivably vary seasonally. This was not accounted for in the study. When making these comparisons I elected to express component-specific IgE concentrations as percentages of the total IgE detected in the sera or bronchial biopsies. Although there is considerable evidence that total and allergen-specific IgE production are regulated independently[220], it was felt that expression of data in this way would best obviate possible variation relating to the cellular content of individual biopsies (since IgE in the tissues is likely to be bound almost exclusively to cells) as well as any possible losses during extraction.

Using the ISAC chip, which is clearly capable of detecting IgE against components of common aeroallergens in the bronchial biopsies, I failed to detect specific IgE against any allergen component in both the sera and the bronchial biopsies of the non-atopic patients, whether asthmatic or not. These data do not therefore support the hypothesis that allergen-specific IgE is sequestered in the bronchial mucosa of non-atopic asthmatics but fails to appear in the circulation. Rather, they suggest that elevated local bronchial mucosal IgE production in non-atopic asthmatics, for which we have produced abundant indirect evidence in previous studies[39,126,218], is directed against targets other than allergens.

Chapter 5: Anti-IgE autoantibodies and their possible effects on the biological functions of IgE *in vivo*

5.1 Introduction

In the original studies described thus far in this work I have shown that disengagement of the IgE molecule from its receptors using anti-IgE has the propensity to improve lung function in asthmatics not regarded as "atopic" by conventional criteria and whose basophils and mast cells consequently apparently lack the ability to mediate Type I hypersensitivity reactions[221] following cross-linking of surface IgE-FcεRI complexes by conventional, multivalent allergens, resulting in activation/degranulation[222] and potentially aggravating clinical symptoms.

Aside from the possibility that non-atopic asthmatics may produce IgE responses to exogenous, multivalent proteins not conventionally regarded as "allergens", another scenario that may at least partly account for the clinical efficacy of anti-IgE therapy in non-atopic asthma is that mast cells and basophils may in some individuals be activated by cross-linking of bound surface IgE by endogenous autoantibodies. Previous studies in humans have suggested that some individuals spontaneously produce autoantibodies of the IgG or IgM class which can bind specifically either to IgE or to FcεRI. Furthermore, some of these autoantibodies have been demonstrated to be capable of activating basophils and mast cells independently of antigens[223], as demonstrated by a variety of *in vivo* and *ex vivo* functional assays such as autologous serum, skin prick tests[224], basophil histamine release[144] and, more recently, basophil activation assays using flow cytometry[225]. Most IgE-specific IgG autoantibodies have been reported to be of the IgG_1 or IgG_4 isotype[226] and appear to recognise epitopes within the IgE Cε2 and Cε4 domains[227]. In contrast, FcεRI-specific IgG autoantibodies of the IgG_1 or IgG_3 isotype have been described predominantly in some patients with chronic spontaneous urticaria,

while autoantibodies of other isotypes such as IgG_2 and IgG_4 have been described in association with other autoimmune diseases such as lupus[228]. Autoantibodies against IgE or FcεRI have been detected in various diseases not generally regarded as "autoimmune", including atopic dermatitis[229,230], asthma[231] and others[228,232].

Interestingly, not all of these autoantibodies show potential pro-inflammatory activity[233,234], at least as detected by the aforementioned assays so that, as with IgE itself their activities do not reflect their local concentrations[235]. Furthermore, IgE-specific and FcεRI-specific autoantibodies are also detectable in apparently healthy individuals[236,237], raising the possibility that they may in some situations actually act in an anti-inflammatory or regulatory capacity. The prior existence in some patients of autoantibodies with these properties may also at least partially explain why exogenous anti-IgE therapy exerts only a marginal additional clinical benefit.

In order to investigate these possibilities further I set out to address the hypothesis that some non-atopic asthmatic patients produce anti-IgE autoantibodies which have the propensity to activate or inhibit target cells in an allergen-independent fashion. For the purposes of practicability and convenience, blood basophils from these patients were chosen as target cells, and the experiments were designed to compare responses in both atopic and non-atopic asthmatic patients as well as non-atopic controls.

I undertook this work in discussion and collaboration with a number of colleague laboratory scientists who helped develop, in many cases *de novo*, the protocols of the *in vitro* experiments. I supplied the serum samples from the patients whom I had

characterised and also participated in the performing of the laboratory work and discussion of the data analysis. What we achieved was:

1. Development and calibration of an *in vitro* assay to detect and quantify IgG anti-IgE autoantibodies in the serum samples from the patients and normal volunteers;
2. Assessment of the capacity of these sera to activate or inhibit IgE-sensitised blood basophils from a single atopic donor in the presence and absence of allergen;
3. Assessment of the capacity of these sera to inhibit the binding of IgE-sensitised basophils to specific allergen using a rat basophilic cell line stably expressing human FcεRI bound to in-house manufactured monoclonal IgE directed against the *Phl p7* component of Timothy grass allergen[194].

5.2 Participants and clinical interventions

17 atopic asthmatics, 19 non-atopic asthmatics and 14 non-atopic non-asthmatic control subjects (non-atopic controls) were recruited based on the definitions and criteria described in chapter 2, under sections 2.1, 2.2, 2.3, 2.5 and 2.6 (2.6.1, 2.6.2 & 2.6.3). 2.7.1.2, 2.7.1.3 and 2.7.4 (2.7.4.1, 2.7.4.2, 2.7.4.3, 2.7.4.4 & 2.7.4.5). Their demographics, baseline characteristics and spirometric data are summarised in Table 5.1. A majority of the non-atopic asthmatics (17/19; all except subjects NAA 18 & 19) were also participants in the omalizumab study described in Chapter 3. Their serum samples were collected before they were commenced on the trial medication (omalizumab or placebo).

5.2.1 Participants

Subject group (n)	Non-atopic Controls (14)	Non-atopic Asthmatics (19)	Atopic Asthmatics (17)
Age (yr)	24 (21-36)	49 (21-70)	31 (24-66)
Sex M/F	8/6	9/10	10/7
Blood eosinophil count ($x10^9$ / L)	0.1 (0-0.4)	0.2 (0-1)	0.3 (0.1-1.1)
FEV1 (% predicted)	106 (94-128)	73 (40-119)	96 (54-119)

Table 5.1: Demographics, baseline characteristics and lung function (FEV_1%) of the study subjects. Age, peripheral blood eosinophil count and FEV_1% predicted are expressed as the median (range)

5.2.2 Anti-IgE autoantibody experiments

The collection of peripheral venous blood, separation of sera and the isolation of PBMC are described in chapter 2 under sections 2.5, 2.7.1.2 and 2.7.1.3. ELISA, Basophil Activation Test and Flow cytometry were conducted as described under section 2.7.4 (2.7.4.1, 2.7.4.2, 2.7.4.3, 2.7.4.4 and 2.7.4.5).

5.3 Statistical analysis

Statistical analysis was performed using Two-Way ANOVA with Bonferroni correction or as otherwise stated. A p value of < 0.05 was considered significant.

5.4 Results

5.4.1 Identification of IgG class autoantibodies against free and FcεRI-bound IgE

An in-house, custom ELISA developed by my colleague Dr Chan was used to screen for IgG class, anti-IgE antibodies in the serum samples as described in the common methods chapter, sections 2.7.4 and 2.7.4.1. All subjects in all three groups had detectable IgG anti-IgE autoantibodies in their sera at concentrations in excess of the threshold of sensitivity of the assay after allowing for non-specific binding, and ranging from 22-223 ng/mL, 11-1761 ng/mL and 39-1070 ng/mL in the non-atopic controls (NAC), non-atopic asthmatics (NAA) and atopic asthmatics (AA) respectively (Figure 5.1A).

Overall, the median concentrations of the IgG anti-IgE autoantibodies in the atopic and non-atopic asthmatics were higher than that observed in the control subjects, achieving statistical significance in the non-atopic, but not the atopic patients (Figure 5.1B). Of course, the pathophysiological significance, if any, of these findings is at present uncertain since all of the healthy controls had detectable autoantibody, so that a "normal" range for these measurements has yet to be defined. Concentrations of IgG anti-IgE were in excess of the 95% confidence limit of the measurements in the normal controls in the serum samples from some of the asthmatic subjects (Figure 5.1A). Although the numbers of these patients were relatively small, the median blood eosinophil counts and lung function (FEV_1% predicted) of the asthmatic patients (both atopic and non-atopic) with circulating concentrations of anti-IgE autoantibodies in excess of the 95% confidence limit of the range seen in the non-asthmatic controls were not significantly different from those with lower concentrations within each group (Figure 5.2, Table 5.2). Taking the study subjects as a whole, no correlation was observed between the concentrations of IgG

anti-IgE antibodies and those of total IgE or anti-nuclear autoantibodies (Figures 5.7 & 5.8).

An interesting property of the IgE-specific autoantibodies was that they appeared to bind equivalently to immobilised, free IgE and FcεRI-bound IgE (Figure 5.3) (with the proviso that FcεRI-bound IgE binding was below the limit of detection of the assay in two serum samples from the non-atopic asthmatics NAA9 and NAA17).

In order to calibrate the standard concentration curve for the IgG anti-IgE antibodies, we used known concentrations of omalizumab, which is of course a synthetic IgG anti-IgE antibody, as a standard for the IgE-binding antibodies (Figure 5.4). Since omalizumab does not bind to FcεRI-bound IgE, we utilised purified, IgG anti-IgE autoantibody from a single patient's serum as a standard to calibrate the ELISA for measuring FcεRI-bound IgE (Figure 5.5). We excluded the possibility of IgE contaminating these samples following our purification procedure by gel electrophoresis (Figure 5.6).

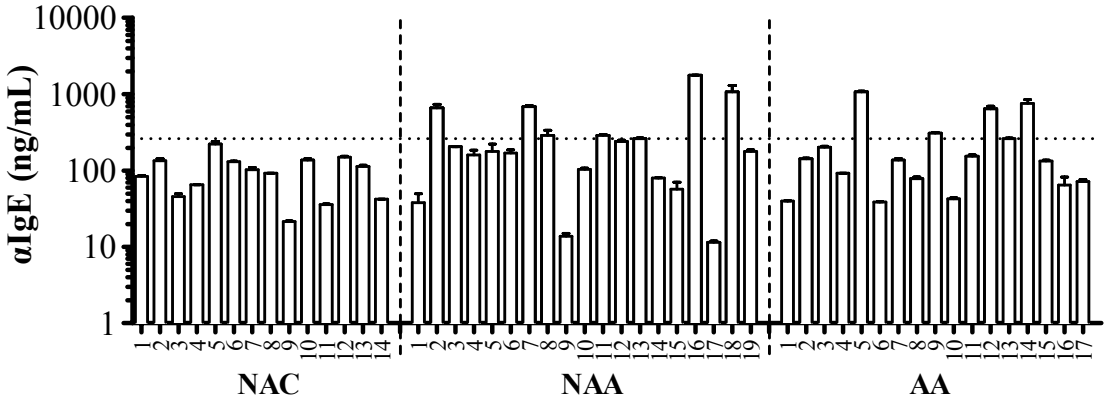


Figure 5.1A: Concentrations of IgG anti-IgE autoantibodies in sera from non-atopic controls (NAC), non-atopic asthmatics (NAA) and atopic asthmatics (AA). Dotted line shows 95% confidence limit of the range in controls. Bars represent the mean/SD of at least three independent experiments.

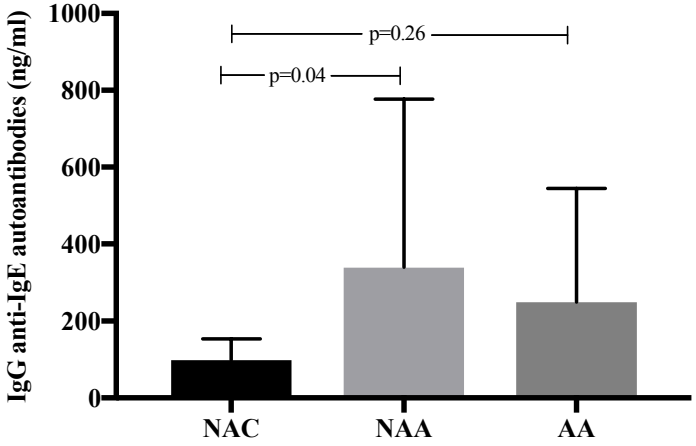


Figure 5.1B: Comparison of IgG anti-IgE autoantibodies in sera from non-atopic controls (NAC), non-atopic asthmatics (NAA) and atopic asthmatics (AA). Bars represent mean and SD.

Subjects		Eosinophil count ($x10^9$/L)	FEV1%
NAA	2	0.6	80
	7	0.4	79
	16	0.2	119
	18	0.2	73
AA	5	1.1	130
	12	0.5	90
	14	0.5	98

Table 5.2: Some baseline characteristics of the subjects with anti-IgE autoantibodies in excess of the 95% confidence limit of the range in controls.

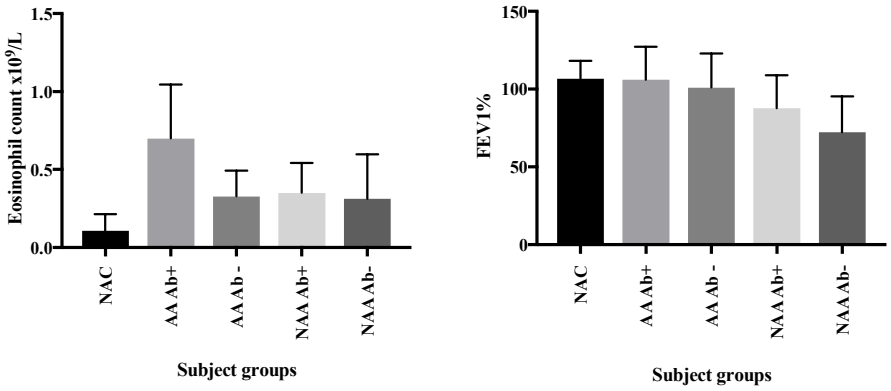


Figure 5.2: Comparison of baseline clinical characteristics (blood eosinophil counts and FEV1% predicted) between the study groups (NAC: non-atopic controls; AA & NAA Ab+: atopic asthmatics and non-atopic asthmatics with anti-IgE autoantibody concentrations in excess of the 95% confidence limits of those observed in the NAC; AA & NAA Ab- : atopic asthmatics and non-atopic asthmatics with anti-IgE autoantibody concentrations within the 95% confidence limits of those observed in the NAC.

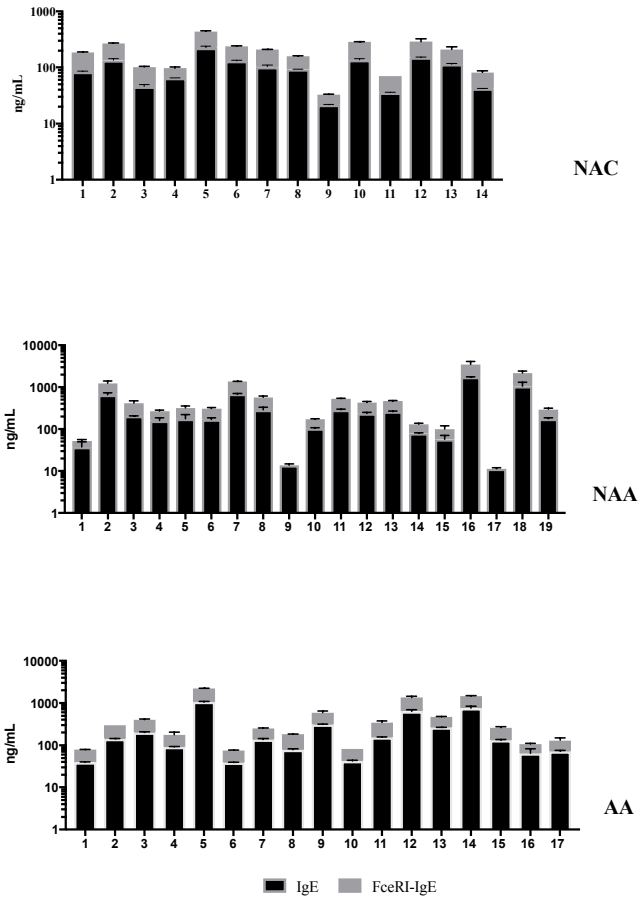


Figure 5.3: The capacity of IgE-specific IgG autoantibodies to bind to "free" IgE (dark bars) and FcεRI-bound IgE (grey bars). NAA 9&17 analytes below detection limit of assay. Bars show mean/SD of three experiments using duplicate samples.

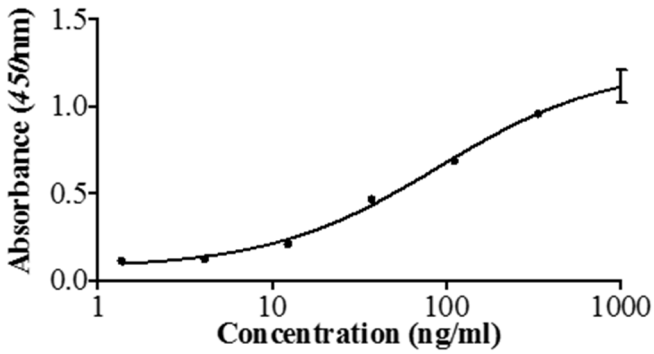


Figure 5.4: Representative standard concentration curve for the IgG anti-IgE ELISA using omalizumab

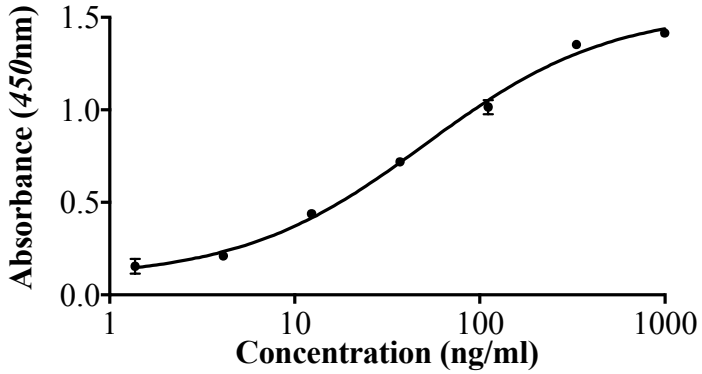


Figure 5.5: Representative standard concentration curve for the IgG anti-FcεRI-bound ELISA using anti-IgE purified from a single human serum sample.

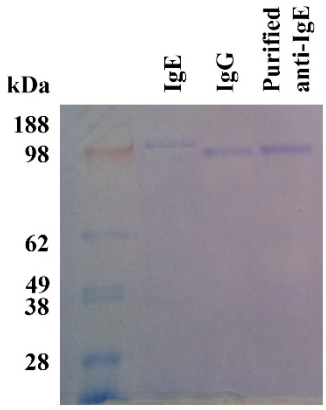


Figure 5.6: SDS-PAGE showing the purified IgG anti-IgE from serum compared with recombinant IgE and IgG.

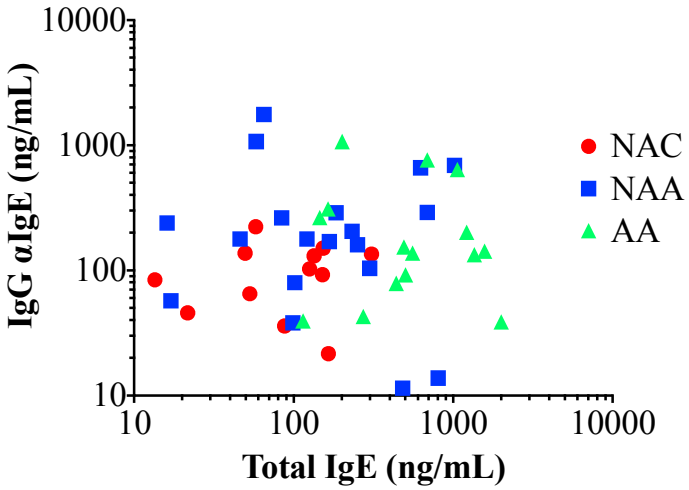


Figure 5.7: Comparison of serum total IgE and IgG anti-IgE autoantibody concentrations in all study subjects. Spearman r = 0.08578, p>0.5.

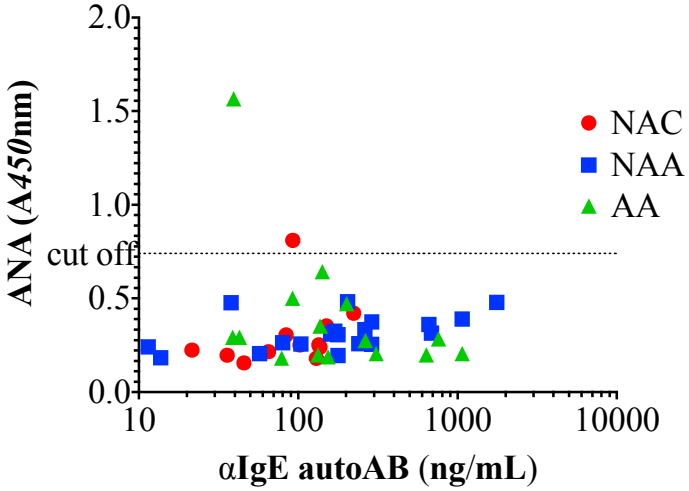


Figure 5.8: Comparison of serum concentrations of IgG anti-IgE antibodies with anti-nuclear autoantibodies (ANA) in all study subjects. Dotted line shows the threshold for a positive ANA result. Spearman r = -0.02621, p>0.5.

5.4.2 Basophil activation by sera containing IgG anti-IgE autoantibodies

A conventional, flow cytometric basophil activation assay was used to test the ability of sera containing IgG anti-IgE specific autoantibodies to activate blood basophils isolated from a single atopic donor in an attempt to create some uniformity in the properties of the responder basophils (at least in the sense that they were derived from the same donor). In order to establish a positive control we incubated these donor basophils with polyclonal, exogenous IgG anti-IgE at a range of concentrations covering that observed in the sera of the study subjects. As expected, this resulted in concentration-dependent activation of the donor basophils as assessed by elevated expression of the surface activation marker CD63 (from 3.15% to 85.6%; Figures 5.9 and 5.10). Having established this standard curve, the same donor basophils were incubated under identical conditions with serum samples from the study subjects. We defined basophil "activation" in this situation as >2.67% CD63+ cells, equivalent to 3 standard deviations above the mean percentage of basophils expressing CD63 following incubation with the donor's own serum under identical conditions. Using these criteria, five of the serum samples from the study subjects were able to activate basophils, interestingly from both the non-atopic (NAA7, NAA18) and the atopic asthmatics (AA5, AA8, AA14) (Figure 5.11). Not all of the basophil-activating sera contained particularly high concentrations of IgG anti-IgE (Figure 5.1A: AA8) and, conversely, not all of the serum samples with particularly elevated IgG anti-IgE concentrations activated basophils (Figure 5.1A: NAA2, NAA16, AA12): in other words, the ability of these sera to activate basophils did not equate with their absolute IgG anti-IgE autoantibody concentrations.

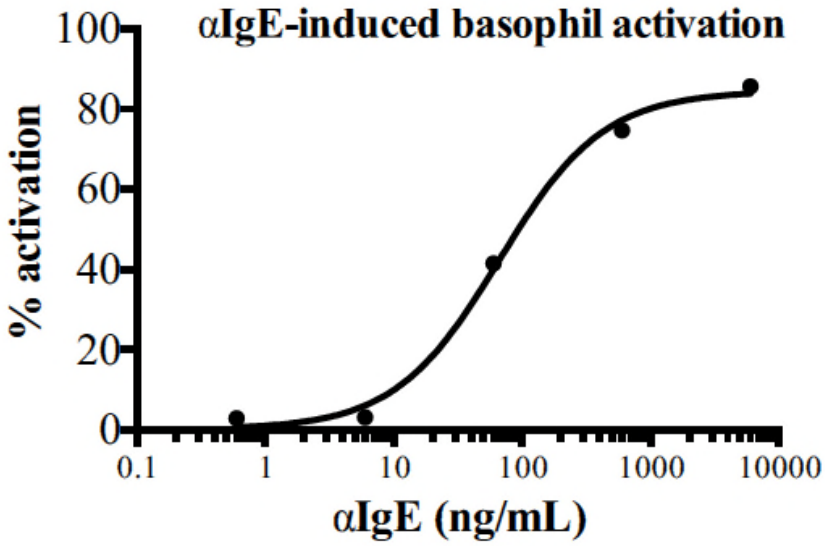


Figure 5.9: Response of blood basophils to polyclonal anti-IgE *in vitro*.

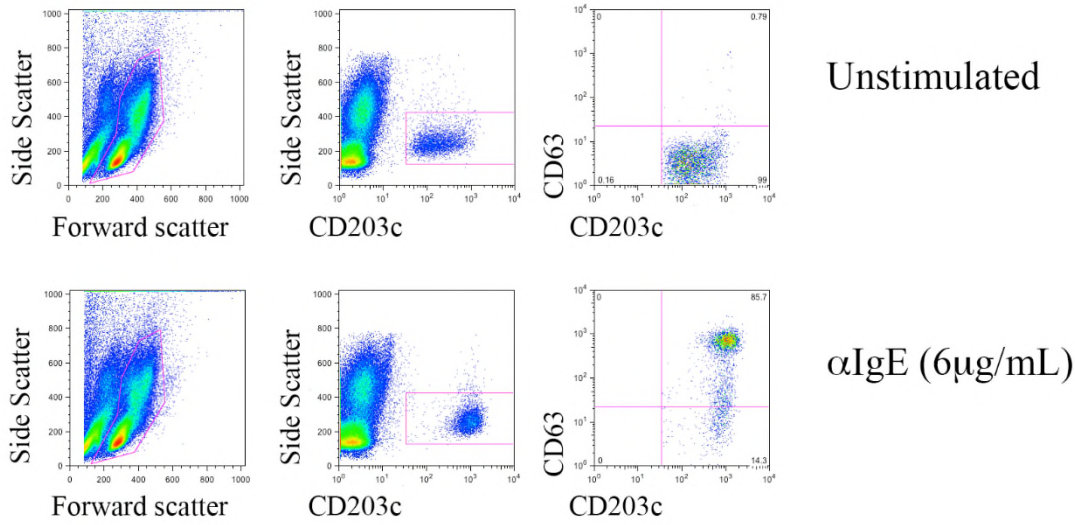


Figure 5.10: Gating strategy to determine basophil activation by flow cytometry, comparing unstimulated and anti-IgE-stimulated PBMC.

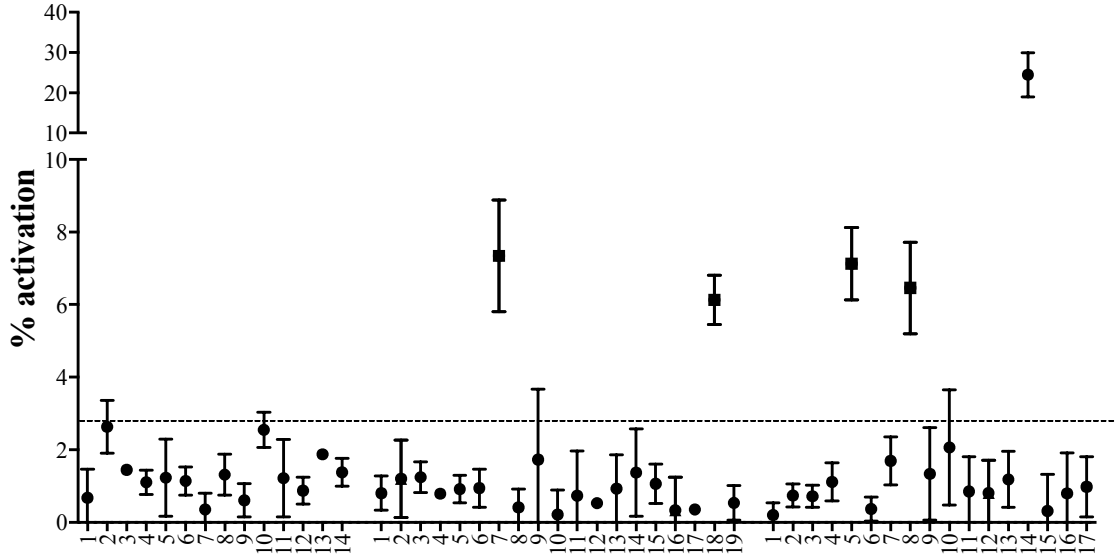


Figure 5.11: Basophil activating activity of sera. Dotted line shows detection threshold. Points with error bars represent the mean/SD of at least three independent experiments.

5.4.3 Modification of allergen-induced basophil activation by IgG anti-IgE autoantibodies

We were finally interested to examine the ability of the sera from our study subjects containing IgG anti-IgE autoantibodies to modify conventional, allergen-induced basophil activation in a suitably sensitised, atopic subject. For this purpose we used blood basophils from a single atopic donor sensitised to a major allergen of house dust mite (*Der p2*). As anticipated, incubation of this donor's blood basophils in the presence of donor serum with *Der p2* induced concentration-dependent activation (Figure 5.12). When pre-incubated with the same donor basophils under identical conditions, none of the five basophil-activating sera identified in the previous section significantly altered the degree of basophil activation induced by a concentration of *Der p2* optimal for this donor (Figure 5.13) as well as a range of higher and lower concentrations of the allergen (Figure 5.14: serum from subject AA14 could not be analysed further because of the paucity of the sample). Fascinatingly, though, we did observe significant inhibition of allergen-induced basophil activation in the presence of sera from two other study subjects (NAA16 and AA12) which in isolation did not directly increase basophil activation, while serum from a third subject (NAA2) exhibited a similar inhibitory effect of borderline significance (Figures 5.14 and 5.15).

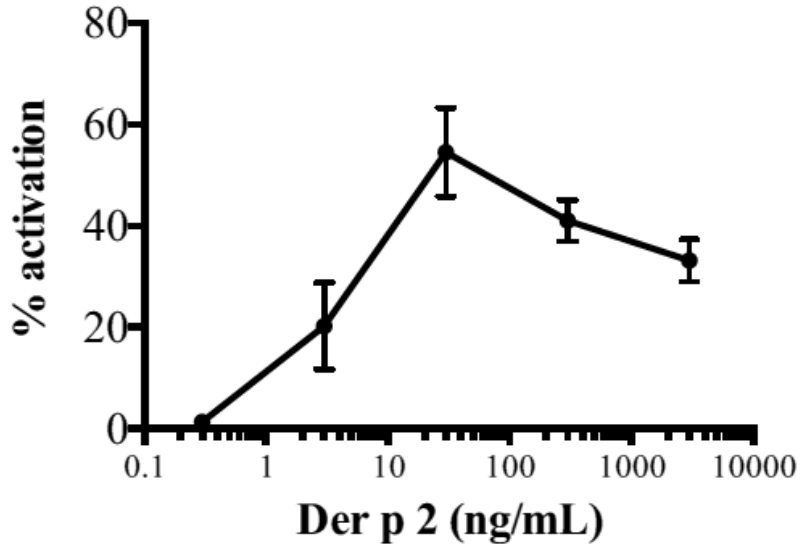


Figure 5.12: Concentration/response curve of blood basophils from a *Der p2*-sensitised atopic donor to *Der p2* allergen *in vitro*.

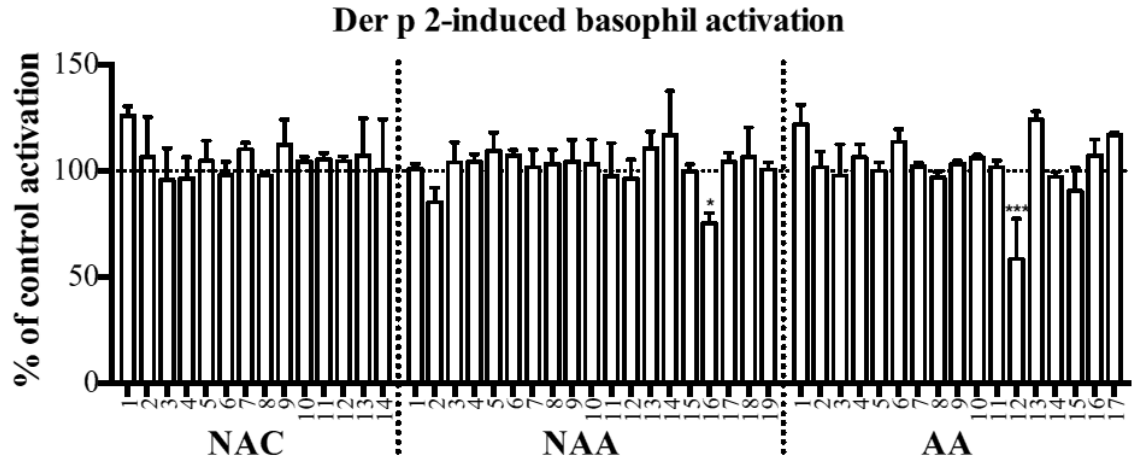


Figure 5.13: Response of the same basophils to *Der p2* 30 ng/mL pre-incubated with all sera from Figure 5.1 normalised to baseline (pre-incubation of the cells with the donor's own serum). Bars represent the mean/SD of at least three independent experiments.

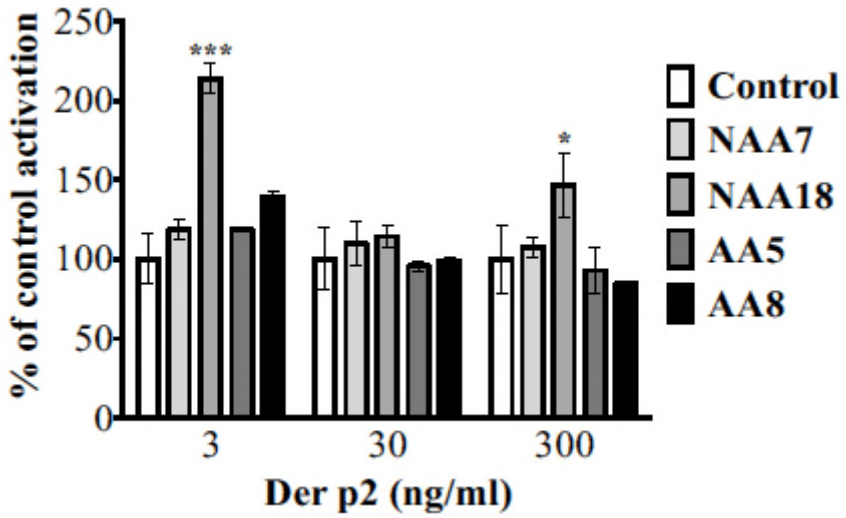


Figure 5.14: Response of blood basophils stimulated with *Der p2* (3-300 ng/mL), pre-incubated with four sera containing IgG anti-IgE autoantibodies and basophil activating activity.

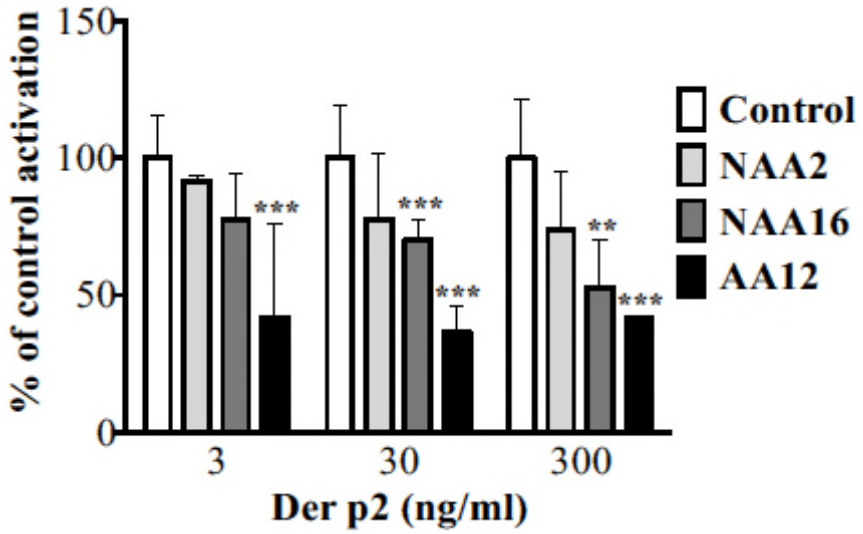


Figure 5.15: Response of blood basophils stimulated with *Der p2* (3-300 ng/mL), pre-incubated with three sera containing IgG anti-IgE autoantibodies without basophil activating activity normalised to baseline (pre-incubation of the cells with the donor's own serum).

5.4.4 Depletion of total IgG and IgE-binding proteins from inhibitory sera abolished inhibition of allergen-induced basophil activation

As a further confirmation of the identities of the inhibitory species identified in the preceding section, we depleted either total IgG (Figure 5.16A) or total IgE-binding proteins (Figure 5.16B) from the sera of subjects NAA16 and AA12 and then repeated the experiments to examine their effects on allergen-induced basophil activation. Both of these manipulations abolished the ability of the sera to inhibit allergen-induced basophil activation, whereas purified IgE binding proteins, when depleted, retained this activity in full (Figures 5.16C and 5.16D). Similar extracts from the non-inhibitory serum NAA7 showed no significant effects on allergen-induced basophil activation (Figure 5.17) which, in acting as a negative control, demonstrated that depletion of IgG or isolation of IgE-binding proteins did not generate basophil-modifying activity *per se*.

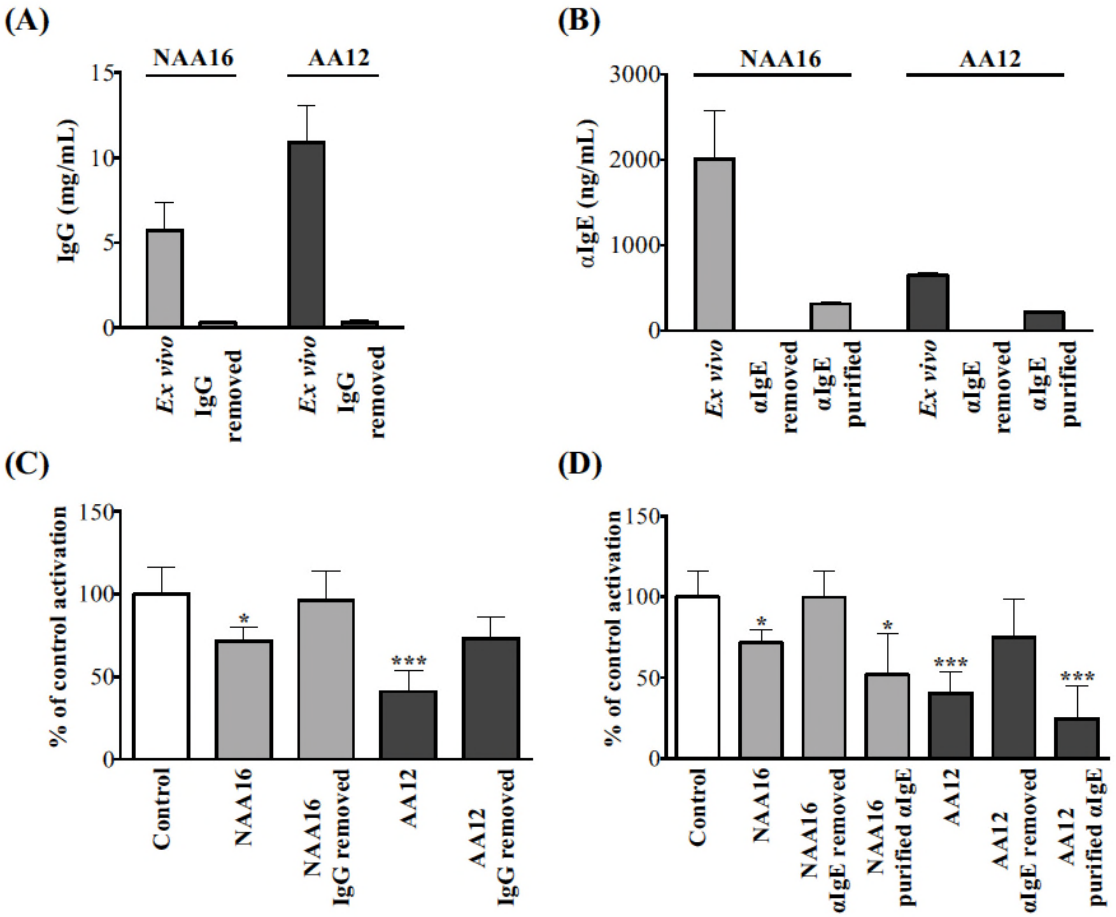


Figure 5.16: (A) Total IgG concentrations in two test sera *ex vivo* and following protein G depletion. (B) IgE-binding proteins *ex vivo* and following depletion and isolation of IgE binding proteins. Effects of sera *ex vivo* and following (C) IgG depletion and (D) removal and purification of IgE binding proteins on *Der p2* (30 ng/mL)-induced basophil activation normalised to baseline (using the donor's own serum). Bars represent the mean/SD of three independent experiments.

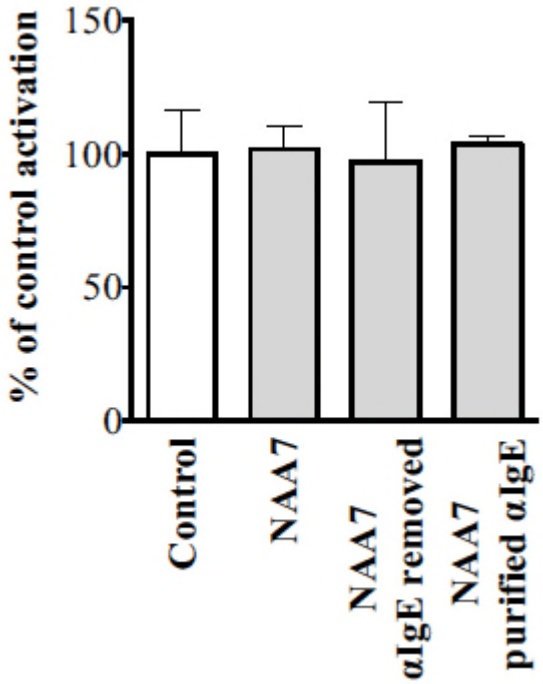


Figure 5.17: Effects of the serum from NAA7 (non-inhibitory) on Der p2 (30 ng/mL)-induced basophil activation before and after removal and purification of IgE binding proteins. (Mean/SD of three independent experiments).

5.4.5 Allergen binding to IgE is inhibited by IgG anti-IgE antibody containing sera

We then wished to investigate possible mechanisms of inhibition of allergen-induced basophil activation by the inhibitory sera NAA16 and AA12. To achieve this we took advantage of an in house system employing the rat basophilic cell line RBL-SX38, which expresses surface human FcεRI, and recombinant IgE specific for the Timothy grass allergen *Phl* p7. First, we confirmed using flow cytometry that the recombinant IgE anti-*Phl p7* binds to the FcεRI expressed on the surface of the RBL-SX38 cells (Figure 5.18A). Then we showed that subsequent addition of fluorochrome-tagged, recombinant *Phl p7* to the IgE-coated cells resulted in its binding to this specific antibody (Figure 5.18B). Pre-incubation of the IgE-bound RBL-SX38 cells with four of the sera which activated basophils directly *ex vivo* but which did not modify allergen-induced basophil activation *ex vivo* (NAA7, NAA18, AA5 and AA8, Figure 5.12: we were unable to re-test serum AA14 owing to paucity of sample), or one serum sample from a donor in each group selected to contain anti-IgE at the threshold of the mean concentration observed in the controls with no basophil activating activity (NAC5, NAA11, AA9) prior to the addition of *Phl p7* did not significantly alter surface IgE or *Phl p7* binding (Figures 5.18C, 5.18D). In contrast, two of the sera which inhibited allergen-induced basophil activation *ex vivo* (NAA16 and AA12, Figure 5.15) significantly reduced binding of recombinant allergen, but not allergen-specific IgE to the RBL-SX38 cells (Figures 5.18C, 5.18D).

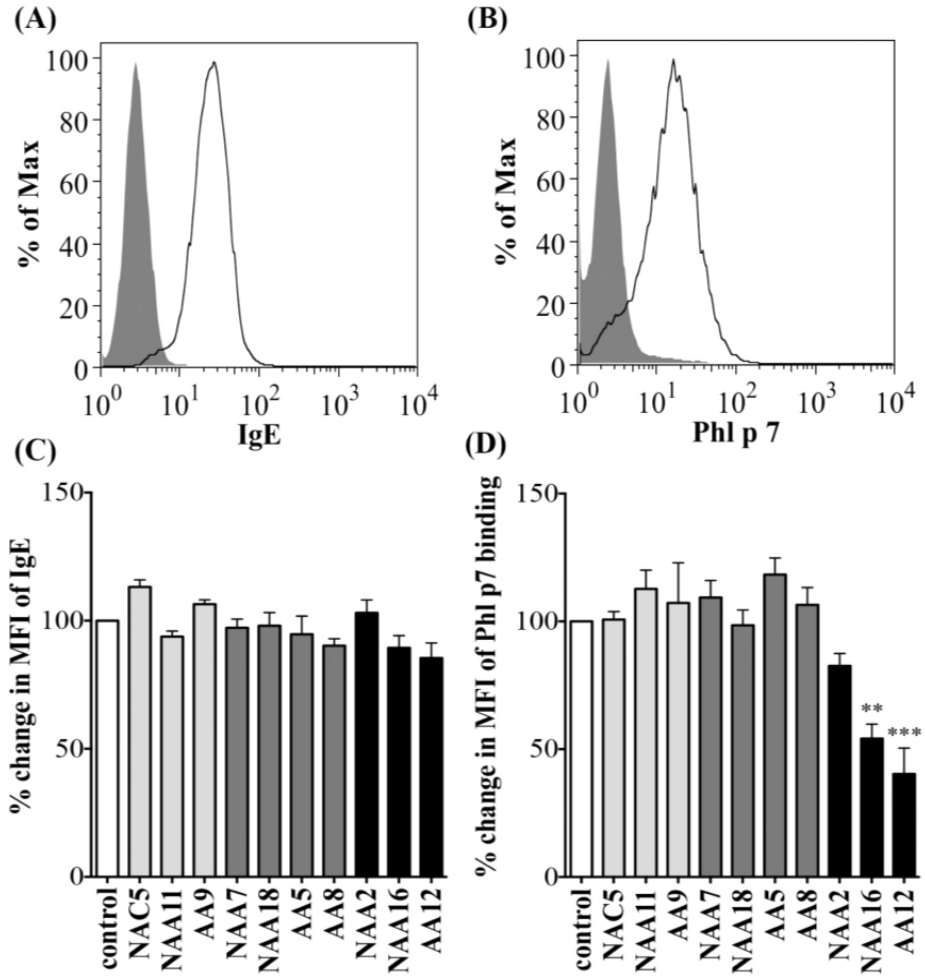


Figure 5.18

(A) Binding of recombinant *Phl p7*-specific IgE to FcεRI on RBL-SX38 cells. (B) *Phl p7* binding following further incubation with specific allergen (representative of three independent experiments). Changes in (C) surface bound IgE and (D) surface bound *Phl p7* on RBL-SX38 cells pre-incubated with recombinant, anti-*Phl p7* IgE, test sera then *Phl p7* compared with no serum control. Bars represent the mean/SD of three independent experiments.

5.5 Discussion

Using the experiments described in this chapter, which were conceived and carried out entirely in house, we were able to develop an entirely new technique for the quantitation of serum IgG anti-IgE autoantibodies. Previously these have been identified only indirectly using histamine release assays[143], or as arbitrary optical density readings in uncalibrated ELISA[226,238]. We succeeded in calibrating our assay using known concentrations of omalizumab, a monoclonal IgG anti-IgE antibody, recombinant IgE as the capture antigen and a pan-anti-human IgG polyclonal antibody for detection.

Using this assay we have been able to show that, in a subset of asthmatics, and regardless of their conventional atopic status, it is possible to detect circulating IgG anti-IgE autoantibodies in excess of the range observed in a group of non-diseased, control subjects. Although this was arguably a sporadic finding, and accepting that its "pathological" significance cannot be clearly gauged from our experiments since the "normal" range was derived from a limited group of subjects, we nevertheless feel that these observations are of great potential interest from the point of view of a possible role for IgE-mediated mechanisms in asthma, since my additional, personal data from chapter 4 (published) clearly suggest that IgE is expressed to excess in the bronchial mucosa of asthmatics regardless of conventional atopic status[188,239], so that the additional presence of systemic IgG anti-IgE autoantibodies capable of directly activating cells bearing sufficient surface-bound IgE[240] could represent a mechanism for local, allergen-independent disease exacerbation. Equally significantly, however, the data also show that some IgG anti-IgE autoantibodies have the potential actually to inhibit allergen-induced activation of basophils expressing surface allergen-specific IgE, at least partly by inhibiting the binding of this IgE to allergen. This may partly explain why, in this and

previous studies, measured concentrations of these antibodies correlate poorly with their clinical effects[241,242], and also why they do not necessarily cause disease[241]. More fundamentally, though, they may at least partly explain phenomena such as why some atopic subjects who manufacture allergen-specific IgE do not develop allergic disease, and why in some patients with asthma, who may manufacture IgE inhibitory IgG endogenously, exogenously administered IgG anti-IgE therapy, such as with omalizumab, proffers little additional clinical benefit.

These studies do not of course address regulatory mechanisms of IgG anti-IgE autoantibody production in individual subjects. We observed no correlation between the production of these antibodies and the production of other potentially pathogenic IgG autoantibodies such as anti-nuclear autoantibodies (Figure 5.8). Similarly, and in contrast to certain earlier studies[231,243] we observed no correlation in individual subjects between serum total IgE and IgG anti-IgE concentrations (Figure 5.7). This may reflect low subject numbers, interference by IgE-specific autoantibodies with the determination of total IgE using different techniques[244] or a true lack of any relationship.

Although again it might be argued that, as in all similar studies, not all of the properties of these antibodies or their relative incidence in the entire source populations may have been characterised in an unbiased manner, it is noteworthy that in no case was their binding inhibited by prior binding of the IgE to its high-affinity receptor FcεRI, suggesting that these IgG species are able to bind to IgE already attached to its high-affinity receptor *in vivo*. This is in contrast to omalizumab, which binds only free IgE. The findings are congruent with previous studies[227] suggesting that the majority of IgG anti-IgE autoantibodies bind to the Cε2 or Cε4 domains of IgE, and not the Cε3 domain

to which omalizumab binds[245] and which is inaccessible when the IgE is bound to its high- or low-affinity receptor. The fact that complete removal of total IgG or total IgE binding proteins abolished basophil modifying activity, whereas the purified IgE binding proteins retained it, is consistent with the hypothesis that it is entirely attributable to IgG anti-IgE. Furthermore, that fact that such activity did not "appear" in fractionated serum from subjects not showing it *ex vivo* (Figure 5.17) excludes the possibility that it is an artefact of the separation procedure. Nevertheless, these experiments cannot completely exclude the possibility that some basophil modifying activity may have reflected the alternative or additional presence of IgG anti-FcεRI autoantibodies[240,246-248]. The unprecedented potential protective effect of some of these antibodies, however, is of great interest regardless of their precise specificities.

The data in this chapter also provide a precedent that inhibitory IgG anti-IgE autoantibodies could reduce allergen-induced basophil activation in some patients. The antibodies that we were able to identify with this effect did not appear to dissociate IgE from its receptor, a property suggested in previous reports[249-251], although some appeared to be able to prevent allergen binding to surface-bound specific-IgE, presumably without cross-linking it. This could reflect the inability of IgG anti-IgE in the IgG-IgE-FcεRI complex to interact with another IgE molecule on the cell surface for a variety of reasons[252]. Further possible mechanisms of basophil inhibition by these antibodies not explored in the present study are also conceivable, such as cross-linking of FcγRIIb with FcεRI[253-255]. It is worth noting again that the activities of these IgG anti-IgE autoantibodies are quite different from those of omalizumab, which appears to exert its clinical effects simply by preventing binding of IgE to its high- and low-affinity receptors, and has never been described to reduce the intrinsic sensitivity of basophils to

activation: on the contrary, recent experiments[256,257] suggest that sequestration of IgE during omalizumab therapy renders basophils hypersensitive to activation by cross-linking of reduced surface-bound IgE molecules, at least partly by resetting of FcεRI coupled intracellular signalling.

Finally, the production of IgG anti-IgE autoantibodies has been well documented in murine "models" of allergen sensitisation and tolerance[258] and in the course of specific allergen immunotherapy in humans[234,259], which induces a vigorous IgG_4 response that could, at least in theory, include IgG anti-IgE autoantibodies. Indeed, one of these studies on wasp venom immunotherapy[234] linked the elevated production of basophil activating IgG anti-IgE autoantibodies with treatment failure.

In summary, inhibitory IgG anti-IgE autoantibodies may contribute to a natural regulatory mechanism that could conceivably influence the severity and presence/absence of diseases with IgE-mediated mechanisms, including asthma and allergic rhinitis, and the outcomes of therapeutic processes such as allergen immunotherapy and therapy with exogenous IgG anti-IgE. This demands further research.

Chapter 6: Final Discussion

6 Final discussion

6.1 Summary of results

In this project, I have used ImmunoCAP and ImmunoCAP ISAC microarrays for the first time to detect, quantify and specify IgE in the bronchial mucosa of both atopic and non-atopic asthmatics. The total IgE concentration in the bronchial mucosal homogenates was significantly elevated in both the non-atopic and the atopic asthmatics compared with the controls. Allergen component-specific IgE species were detectable in the sera and biopsy homogenates of the atopic asthmatics. I failed to detect, however, specific IgE against any allergen component in both the sera and the bronchial biopsies of the non-atopic patients, whether asthmatic or not, using the ISAC chip.

IgG autoantibodies binding to both free and FcεRI-bound IgE were detected in patients with atopic and non-atopic asthma, as well as controls. While some were able to activate IgE-sensitised basophils, others inhibited allergen-induced basophil activation, at least partly by inhibiting binding of IgE to specific allergen. Although the subject numbers were relatively small and the detection of these antibodies sporadic, these findings are consistent with the hypothesis that naturally occurring IgG anti-IgE autoantibodies contribute to both inhibition and activation of basophils in some individuals, and furthermore in a manner distinct from that of existing therapeutic IgG anti-IgE antibodies such as omalizumab. The propensity of some individuals to make these antibodies may at least partly explain the longstanding enigma of why atopic subjects who make allergen-specific IgE never develop clinical symptoms, and even the observation that omalizumab therapy is of variable clinical benefit in severe atopic asthma (if its potential inhibitory

activity on allergen-induced basophil activation is already being subsumed in some individuals by a naturally occurring autoantibody).

Omalizumab (or placebo) was administered to a group of moderate and severe non-atopic asthmatics in order to examine its effects on lung function and IgE expression in the bronchial mucosa. Compared with baseline, the median changes in absolute and % predicted FEV_1 following 20 weeks of therapy (primary outcome measure) were positive in the omalizumab treated patients despite substantial reduction of existing therapy, but negative in the placebo treated patients. I also observed a significant reduction in the median total IgE^+ cells ($p<0.001$) (co-primary outcome measure), in sections of bronchial mucosal biopsies from the patients treated with omalizumab but not placebo. No significant changes were observed in median numbers of any of the other cells analysed following omalizumab or placebo therapy. Very few of the B cells and plasma cells showed detectable IgE immunoreactivity as expected, so it was impracticable to evaluate changes. Conglomerates of $CD38^+$ plasmablasts and CD20+ B cells were detected in approximately 1 in every 10 sections of the mucosa, but again these were insufficient in number to quantify possible changes.

6.2 Review of hypotheses and final conclusions

The data from analysis of mucosal IgE concentrations shed further light on the presence, quantity and specificity of IgE in the bronchial mucosa in comparison with a distal site. The detection of IgE in the bronchial mucosa of non-atopic asthmatics in quantities equivalent to that of atopic asthmatics supports the hypothesis that IgE synthesis or at least sequestration is ongoing in this compartment in both atopic and non-atopic asthmatics. It also further supports the hypothesis that IgE plays a role in the pathogenesis

or exacerbation of non-atopic asthma. This hypothesis is further supported by additional preliminary reports that anti-IgE therapy is of benefit in some non-atopic asthmatics[207,208,260-263].

In contrast, the failure to detect allergen-specific IgE utilising the ISAC assay suggests that this IgE is not directed against the common allergens represented in the ISAC panel. The most intriguing question here is the possible specificities and functional activities of the IgE detectable in the bronchial mucosa of the non-atopic asthmatics in quantities equivalent to those observed in atopic asthmatics. There is ample precedent that IgE directed against antigens other than allergens may play a role in asthma pathogenesis, for example RSV IgE in child asthma[264] and IgE against enterotoxins of *Staphylococcus*[150] which may themselves induce IgE production by their superantigen activity.

In addition to the possibility that IgE is directed against non-allergen antigens in asthma, whether or not associated with atopy, it is pertinent to note that IgE pathways may be activated by non-conventional means in both atopic and non-atopic asthma, including by IgG autoantibodies directed against IgE or FcεRI[223] and IgE with cytokinergic activity[134], while IgE directed against tissue components has been implicated in the pathogenesis of some diseases associated with atopy such as eczema[142].

There has been a small series of studies which has appeared over the past 30 years suggesting that atopic patients with allergic rhinitis may secrete allergen-specific IgE into the nasal mucosa in quantities which most likely reflect local, rather than systemic production[88,265]. Most recently, Rondon and colleagues have presented evidence[90] that a subset of patients with persistent rhinitis who are non-atopic by conventional criteria

nevertheless manifest acute symptoms in response to nasal aeroallergen challenge and elaborate allergen-specific IgE into nasal secretions: they have termed this “local allergic rhinitis” or “entopy”. The precise origin of this IgE is unknown, but if it reflects local synthesis one might speculate that relatively prolonged, intense allergen exposure, as likely occurs in the upper airways, may sometimes result in local IgE synthesis and further that non-atopic patients may in some circumstances secrete allergen-specific IgE in response to exposure lower in the respiratory tract.

IgG anti-IgE autoantibodies were detectable in the sera of asthmatics and controls irrespective of their atopic status, but there was no significant difference in the concentration of autoantibodies between the groups. This observation was not in agreement with our original hypothesis that these antibodies are elevated in asthmatics compared with the controls. However, further characterisation and functional analysis of these antibodies led to interesting observations. The anti-IgE autoantibodies were distributed non-uniformly among subjects and induced or inhibited basophil activation. It is tempting to speculate that autoantibody production could be a distinctive feature of individual subjects rather than groups, and that the existence of such antibodies may at least partly address the age old enigma of why many atopic subjects with evidence of IgE sensitisation to particular allergens do not manifest symptoms on exposure, and why in some patients, because of the presence of pre-existing, endogenous IgG anti-IgE, the clinical response to omalizumab is not as extensive as it might have been. Conceivably, IgE autoantibody status may become part of the definition of new endotypes of asthma in the future.

Omalizumab therapy of non-atopic asthmatics reduced bronchial mucosal IgE^+ cells and improved lung function despite withdrawal of conventional therapy, favouring my hypothesis. However, there were no significant changes in the numbers of individual inflammatory cells such as eosinophils, plasma cells or B cells between the omalizumab and placebo treated groups, in contrast to a study of atopic asthmatics (interpretation of which is complicated by the fact that these milder asthmatics did not improve, as judged by changes in PC_{20}, following therapy, nor was it clear that the study was powered to detect such a change)[185]. Nevertheless, changes in cell numbers do not necessarily equate with changes in the function of individual cells, especially when these may exist in clusters and are consequently difficult to enumerate. Furthermore, as expounded in the introduction to this thesis (Chapter 1), until the interactions between airways smooth muscle hyperresponsiveness and airways inflammation in regulating airways calibre are more completely understood, the evidence for involvement of any inflammatory cell in asthma pathogenesis must remain circumstantial.

Omalizumab may conceivably reduce mast cell and basophil activation caused by IgE directed against non-allergen antigens or by certain IgG anti-IgE autoantibodies. My data clearly show that allergen-specific IgE (directed against common allergens) is very unlikely to be involved in the pathogenesis of non-atopic asthma, raising the question of how far it is involved in "atopic" asthma: again, does "allergic inflammation" really exist or is it all alarmin driven? Perhaps the only role of allergen-specific IgE in asthma is to exacerbate the disease in the short term on allergen exposure of suitably sensitised individuals.

Omalizumab may restore innate anti-viral immunity, leading to reduced viral epithelial damage with consequent alarmin production and increased airways inflammation, which narrows the calibre of the airways and exacerbates disease. My study has provided preliminary proof of principle that omalizumab can increase the internal calibre of the airways in the relatively short term, in presence of reduction of IgE binding to airways inflammatory cells: cross-linking or binding of IgE to dendritic cells is postulated to cause impaired interferon responses, as shown in the inner city children study[266], in which omalizumab, administered to asthmatic patients in the few months prior to the September epidemic, wiped the epidemic out completely.

6.3 Future directions

The work embodied in this thesis suggests several future directions for further research addressing the role of IgE, and endogenous antibodies directed against IgE in the pathogenesis of asthma and allergic inflammation.

The data clearly justify closer examination of the possible therapeutic worth of anti-IgE therapy in non-atopic asthmatics in larger studies with conventional clinical outcomes, such as the one conducted in inner-city atopic asthmatic children[266].

Clearly there must be further scrutiny of the mechanisms whereby IgE may regulate the severity and stability of asthma, enabling more substantial appreciation of the likelihood that any given patient will respond clinically to anti-IgE therapy using criteria which extend far beyond the borderlines of conventional atopic status. ‘Respiratory virus exacerbated asthma’ might potentially be considered as a new asthma endotype in future; the role of IgE in determining the consequences of viral exacerbations of disease and the

role of anti-IgE in the treatment of virally exacerbated asthma would also become an area of research interest. However, virus exacerbation is such a common phenomenon across all types of asthma and defining a subgroup based on the criterion of 'viral exacerbation' may not be straightforward due to the considerable overlap of various subtypes.

Further consideration should be given to the possible contribution of antigens other than aeroallergens, and unconventional mechanisms of activation and inhibition of IgE signalling in the pathogenesis of atopic and non-atopic asthma.

Notwithstanding the findings of this study suggesting that IgE production is not centered in the bronchial mucosa of asthmatics, it would clearly be of enormous importance to pinpoint where such production is indeed centralised, if anywhere. This would require more detailed comparison of bulk IgE production at various mucosal surfaces and in the reticuloendothelial and lymphatic systems, as well as a deeper understanding of the extent of IgE trafficking between compartments.

Finally, it would be fascinating to investigate the hypotheses that endogenous IgG, anti-IgE antibodies may at least partly underlie the lack of clinical responsiveness to allergen in some atopic patients, limit the clinical responsiveness of some asthmatics to exogenous anti-IgE therapy (such as with omalizumab) and contribute to the clinical effectiveness of allergen immunotherapy.

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Appendices

Appendix 1: Standard Operating Procedure for ImmunoCAP for the estimation of total IgE

STANDARD OPERATING PROCEDURE

SOP #: C-09

Allergy Medicine Risk Assessment - Protocol / Method

Name of procedure: Standard Operation Procedure (SOP) to run total IgE experiment with ImmunoCAP 100^{E}		
Name of person carrying out risk assessment: Mohamed Shamji **This standard Operating Procedure has be written by Janice Anastasia**	Date: 12/01/2011	Date for next review:

Protocol:

Purpose

This protocol for ImmunoCAP Total IgE provides an in vitro test for quantitative measurement of the total amount of circulating IgE in human serum samples. IgE Antibodies are produced during sensitization to allergens and measurement of this circulating total IgE will aid in clinical diagnosis of IgE-mediated allergic disorders. Increasing levels of total IgE is usually observed in patients suffering from asthma and hay fever.

Health and Safety Guidelines

Safe laboratory practices should be carried out at all times. Prior to commencing this or any laboratory tests, all staff will have read the Health and Safety SOP and discussed any questions/issues arising from it with a senior member of staff.

PROTECTIVE EQUIPMENT

CLOTHING Fastened Lab coat

DISPOSABLE GLOVES Nitrile Gloves

EYE PROTECTION - not required

STANDARD OPERATING PROCEDURE

SOP #: C-09

Buffers & reagents:

Washing Solution prepared from Purified water (ddH_2O), Washing Solution Concentrate, Washing Solution Additive (Phadia)

Rinse Solution prepared from ddH2O

STANDARD OPERATING PROCEDURE

SOP #: C-09

ASSAY PROCEDURE

1. To start up IDM (ImmunoCAP 100^{E}) software and to run allergen specific IgE assay

   1.1 Turn on the ImmunoCAP 100 machine and the computer. Click on the shortcut icon "IDM Software" on the desktop to start the software.Alternatively, select "Start - Programs – IDM Software –IDM Software" in the Windows Start menu.

   1.2 The IDM workspace appears, and the main application components appear in the workspace

   1.3 On the remote computer select on the Request screen (F2). This shows all samples that has been analysed (status as 'approved') or the newly added ones (with status as 'not started'

   1.4 Click on 'New' to add a new sample. Enter the name of the sample in the field 'Sample ID' and other relevant details. Press 'save'.

   1.5 Click on 'Add test' to select the relevant assays to be run on the sample. Select 'tIgE' for total IgE measurements then select the allergen of interest (i.e.G6 (Timothy grass – *Phleum pratense*) Art. No. 14-4100-01). Click on 'add' and select another test. When finish, click 'save'.

   1.6 When all samples have been added, press 'Import' twice to return to the Home screen.

   1.7 Click on the instrument required on the Home screen and the Run Setup screen will appear. Click on Not Started and on Assay. Select the Samples added earlier and select 'tIgE' to measure total IgE values.

   1.8 Tick on the 'curve controls' and the selected assay run can now be sent to the instrument by selecting Send. The IDM computer will show a message, "Send Run Setup to Instrument?" Press, yes. Upon the message "The Run Setup was loaded in the instrument", press ok.

   1.9 The Run setup window should then be closed by pressing Back to prevent duplicate tests being created. Run setup then continues on the instrument.

   1.10 Prepare reagents and refer to the ImmunoCAP 100 Instrument for further instructions on loading the samples and reagents and starting the run.

2. To Run a Calibration Curve Assay (every 28 days or when a new lot number of conjugate or ImmunoCAP is introduced).

   2.1 Follow Step 1.1 to 1.6

   2.2 In the Home screen, instead of ticking 'curve controls', tick '**Calibration Curve**'. The selected calibration curve assay can now be sent to the instrument by selecting **Send**.

   2.3 The selected assay run can now be sent to the instrument by selecting **Send**. The Run setup window should then be closed by pressing **Back** to prevent duplicate tests being created. Run setup then continues on the instrument.

2.4 Prepare reagents (Allow reagents to come to room temperature) and refer to the ImmunoCAP 100 Instrument for further instructions on loading the samples and reagents and starting the run (described in section 3.4).

3. To Run a new assay: Load and Start the ImmunoCAP 100 machine

3.1 In Prepare Run mode press Yes.

3.2 In Enter Request mode press 3

3.3 *"Load and Start"* - Press Yes.

3.4 *"Load Samples"* – Press Yes. Remove the caps on the reagents/ samples before placing them in the Sample Carousel. Make sure that there is enough volume to be withdrawn. Check for air bubbles or film on the surface of the reagents/samples. If there are air bubbles or film, pinch them away. Otherwise the pipette sensor will not detect the surface properly.

3.5 *"Wash/Rinse/Waste OK?"* - Check that the Washing, Rinse and Waste bottles are loaded. Press Yes to confirm this step.

3.6 *Add all the required samples with the required volumes* - Follow the ImmunoCAP 100 Instrument software instructions for sample and loading positions on the sample carousel. Press **Enter** after placing each sample on the carousel to move to the next position. Press **Enter** until all solutions are loaded.

3.7 *Add all the required controls with the required volumes* - Press **Enter** until all controls are loaded.

3.8 *"Load Reagents?"* - Press **Yes** to load the reagents.

3.9 *Add all required reagents (i.e.total IgE conjugate) with the required volumes* – Follow the ImmunoCAP 100 instrument instructions and place the appropriate reagents in the requested position of the carousel. Press Enter to confirm this step.

3.10 *Add all the required reagents with the required volumes* - e.g. Development Solution, Stop Solution, etc in the given positions. Press **Enter** until all the solutions are loaded.

3.11 Press **Yes** to start loading ImmunoCAP /EliA Wells.

3.12 Press **enter** to start a blank check.

3.13 After the blank check is finished start loading the appropriate ImmunoCAP /EliA Wells in the dispenser when requested.

3.14 Type the Lot Specific Code that is found on the label on the EliA Well foil bag and on the EliA Well Carrier. Press **Enter** to confirm the Lot Specific Code.

3.15 *"All Loaded. Remove Carrier. Press Enter."* - When all ImmunoCAP/EliA Wells are loaded, remove the carrier. A message tells you that everything is loaded. Press **Enter** to confirm this step.

3.16 *"Start Process"* - Press **Yes** to be able to start processing the assay run. If you press **No** you will return to the Load Reagents mode.

3.17 *"Press Enter To Start The Process"* - To start the process press **Enter**.

4. Results

4.1 IDM is configured to get results automatically when the run is ready. Press Result button (F3). At the top of the screen Select Instrument, and Select View for either Assay Run or Analytical Run

4.2 Highlight the run for which you wish to view results and press Open. In the result window, 6 tabs are available

4.3 To print results: Results can be printed when in any of the 6 tabs of the results screen, by pressing the Print button at the top of the screen. A box will appear saying the following: Include calibrators – option to print active calibration curve with results. Include lot numbers alimits. Print whole assay run – includes all analytical runs within the assay run.

Appendix 2: Standard Operating Procedure for ImmunoCAP ISAC for the estimation of component specific IgE

CATEGORY:	Laboratory Work Instructions	SOP Number:	
TITLE:	Measurements of specific antibodies to multiple allergen components using Phadia ImmunoCAP ISAC® Micro Array Analysis		

AUTHOR: Janice Anastasia	Signed:
	Title of Author: Research Technician
	Date:

APPROVED BY: Mohamed Shamji	Signed:
	Title: Research and Quality Manager
	Date:

EFFECTIVE DATE:

REVISION HISTORY:			
Number	Section	Pages	Initials/Dates

Measurements of specific antibodies to multiple allergen components using Phadia ImmunoCAP ISAC® Micro Array Analysis

11/01/2011

1.0	Title
	Measurements of specific antibodies to multiple allergen components using Phadia ImmunoCAP ISAC® Micro Array Analysis

2.0	Purpose
	Provide the operator instructions to measure specific antibodies to multiple allergen components using Phadia ImmunoCAP ISAC® Micro Array Analysis.

3.0	Procedures **All work needs to be performed using good clinical and laboratory practice. If unsure please refer to Health & Safety policy.**		
	3.1	**Preparation of specimen, reagents and equipment**	
		3.1.1	Prepare 700 ml of a fresh 1:20 dilution of component A in purified water to obtain Solution A (add 665 ml purified water to 35 ml component A). The volume is calculated for 3 washing steps of 220 ml each, using the washing dishes provided with the starter kit.
		3.1.2	Place a fresh paper towel in the bottom of the humidity chamber and soak it with purified water. Until further use, close the lid of the humidity chamber to prevent evaporation.
		3.1.3	Put ImmunoCAP ISAC into the Washing Dish containing the removable glass slide rack and approximately 220 ml of solution A together with a magnetic stirring bar in the bottom of the dish.
		3.1.4	Place it onto a magnetic stirrer and stir vigorously for 60 minutes.
		3.1.5	Move the glass slide rack containing the ImmunoCAP ISAC into a separate washing dish containing approximately 220 ml purified water.
		3.1.6	Add a stirring bar and stir vigorously on a magnetic stirrer for 5 minutes.
		3.1.7	Remove the glass slide rack containing ImmunoCAP ISAC and place it onto a paper towel and leave to air-dry. Wait until the chips are completely dry.

		3.1.8	Continue with the test procedure described at section 3.2.

	3.2	**Carrying out the ImmunoCAP ISAC Test Procedure**	
		3.2.1	Place the prepared ImmunoCAP ISAC IgE in the Humidity Chamber with the reaction sites facing upwards.
		3.2.2	Pipette 20 µl of each sample onto one reaction site (there are 4 reaction sites available per chip) while avoiding any direct contact of the pipette tip with the surface of ImmunoCAP ISAC IgE. Close the Humidity Chamber carefully, without mixing the samples.
			Note: It is recommended to use one IgE control serum per ImmunoCAP ISAC Assay Kit IgE
		3.2.3	Incubate at room temperature for 120 minutes.
		3.2.4	Remove ImmunoCAP ISAC IgE from the Humidity Chamber carefully, without mixing the samples.
		3.2.5	Remove the samples by tapping the chip with its long side on a fresh paper towel.
			Note: Be careful to shake off the samples at right angles with the glass edge to avoid samples running over neighbouring reaction sites.
		3.2.6	Wash ImmunoCAP ISAC IgE with approximately 220 ml Solution A for 10 minutes (Using the washing Dish and the magnetic stirrer as described previously)
		3.2.7	Move the glass slide rack containing ImmunoCAP ISAC IgE into a washing dish containing approximately 220 ml purified water. Wash for 5 minutes.
		3.2.8	Allow the washed ImmunoCAP ISAC IgE to air dry until completely dry.
		3.2.9	Place the dry ImmunoCAP ISAC IgE in the humidity chamber with the reaction sites facing upwards.
		3.2.10	Discard all used washing solutions.

		3.2.11	Pipette 20 µl of IgE Detection Antibody solution onto each reaction site of the ImmunoCAP ISAC. Make sure that ImmunoCAP ISAC IgE is properly placed in the humidity chamber and close the lid.
		3.2.12	Incubate at room temperature for 60 minutes, protected from light.
		3.2.13	Remove ImmunoCAP ISAC IgE from the Humidity Chamber carefully and remove IgE Detection Antibody sultion by tapping ImmunoCAP ISAC IgE with its long side on a fresh paper towel or rinse gently under distilled water.
		3.2.14	Wash ImmunoCAP ISAC IgE with approximately 220 ml solution A for 10 minutes (using the washing dish and the magnetic stirrer)
		3.2.15	Move the glass slide rack containing the ImmunoCAP ISAC IgE into a washing Dish containing approximately 220 ml purified water. Wash for 5 minutes.
		3.2.16	Discard all used washing solutions.
		3.2.17	Allow the washed ImmunoCAP ISAC IgE to air dry until completely dry.
		3.2.18	ImmunoCAP ISAC IgE is now ready for reading. Use it directly for data acquisition in an appropriate microarray scanner or store dry and protected from light for subsequent reading.

Appendix 3: Peer reviewed publications

Appendix 3.1: “Omalizumab reduces bronchial mucosal IgE and improves lung function in non-atopic asthma”

Omalizumab reduces bronchial mucosal IgE and improves lung function in non-atopic asthma

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ABSTRACT Omalizumab therapy of non-atopic asthmatics reduces bronchial mucosal IgE and inflammation and preserves/improves lung function when disease is destabilised by staged withdrawal of therapy.

18 symptomatic, non-atopic asthmatics were randomised (1:1) to receive omalizumab or identical placebo treatment in addition to existing therapy for 20 weeks. Bronchial biopsies were collected before and after 12–14 weeks of treatment, then the patients destabilised by substantial, supervised reduction of their regular therapy. Primary outcome measures were changes in bronchial mucosal IgE^+ cells at 12–14 weeks, prior to regular therapy reduction, and changes in lung function (forced expiratory volume in 1 s) after destabilisation at 20 weeks. Quality of life was also monitored.

Omalizumab but not placebo therapy significantly reduced median total bronchial mucosal IgE^+ cells ($p<0.01$) but did not significantly alter median total mast cells, plasma cells, B lymphocytes, eosinophils and plasmablasts, although the latter were difficult to enumerate, being distributed as disperse clusters. By 20 weeks, lung function declined in the placebo-treated patients but improved in the omalizumab treated patients, with significant differences in absolute ($p=0.04$) and % predicted forced expiratory volume in 1 s ($p=0.015$).

Omalizumab therapy of non-atopic asthmatics reduces bronchial mucosal IgE^+ mast cells and improves lung function despite withdrawal of conventional therapy.

@ERSpublications
Omalizumab reduces mucosal IgE and improves lung function in non-atopic asthma
http://ow.ly/CHFu3034fcC

Editorial comment in *Eur Respir J* 2016; 48: 1538–1540.

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Conflict of interest: Disclosures can be found alongside this article at erj.ersjournals.com

Introduction

Asthma remains a leading cause of suffering worldwide [1]. In the UK, an estimated 10% of the 5.2 million sufferers remain symptomatic and vulnerable to exacerbations [2]. The humanised, monoclonal IgG_1 anti-IgE antibody omalizumab is the vanguard of an arsenal of new biologicals purposed to improve the lives of severe asthma sufferers. Its clinical effectiveness in a substantial proportion of severe asthmatics is now acknowledged by relevant bodies worldwide, including the British Thoracic Society (BTS) and The National Institute for Health and Care Excellence [3] in the UK.

A major challenge when deploying treatment with biologicals for severe asthma is the possibility of mechanistic variation in the disease, requiring pre-identification of potential responders to any specific agent. In the case of omalizumab, the *prima facie* effect of which is to prevent binding of IgE to its high- and low-affinity receptors [4, 5], the tacit assumption has been that it improves asthma stability fundamentally by reducing or abolishing mast cell and basophil activation by cross-linking of surface-bound allergen-specific IgE by allergen in suitably sensitised, "atopic" patients. Consequently, key clinical trials investigating its efficacy, such as INNOVATE (The INvestigatioN of Omalizumab in seVere Asthma TrEatment (INNOVATE) study) [6] have been limited to atopic asthmatics while its marketing authorisation restricts its use to patients with "convincing IgE-mediated asthma". This phrase is not universally defined but is in practice usually equated with evidence of IgE sensitisation (by skin prick or *in vitro* testing) to one or more common perennial aeroallergens. Conversely, the therapy has been denied to at least, we estimate, 20000 otherwise eligible non-atopic severe asthmatics in the UK, and many more worldwide (the prevalence of non-atopic, severe asthma was estimated at 50% of the total in the ENFUMOSA (European Network For Understanding Mechanisms Of Severe Asthma) study [7] and 17–34% in the SARP (Severe Asthma Research Programme) study) [8].

Much indirect evidence suggests that IgE may play a role in asthma regardless of conventional atopic status. Epidemiologically, asthma was five-fold more prevalent in a cohort of non-atopic subjects with elevated total serum IgE [9]. We and others have shown that atopic and non-atopic asthma are virtually identical in terms of their bronchial mucosal cellular and molecular immunopathology [10–17], evidence of local B cell switching to IgE synthesis [18, 19] and elevated local FcεRI receptor expression [20] and total bronchial mucosal IgE concentrations [21]. Furthermore, IgE directed against antigens other than aeroallergens, such as viral antigens [22] and Staphylococcal enterotoxins [23] has been implicated in asthma pathogenesis. IgE may influence mast cell function by antigen-independent mechanisms [24, 25] and theoretically exacerbate asthmatic bronchial mucosal inflammation by mechanisms not involving mast cells and basophils, such as by enhancing antigen capture [26]. All of these data lend weight to the view that the presence or absence of atopy as operationally defined might not be an appropriate criterion for predicting responsiveness to omalizumab therapy.

Consequently, we elected in a proof-of-concept study to address the hypotheses that omalizumab therapy of non-atopic asthmatics reduces the numbers of IgE^+ leukocytes (B cells, plasmablasts and mast cells) within the bronchial mucosa of the target organ and exerts a favourable effect on lung function in the short term, as assessed by changes in forced expiratory volume in 1 s (FEV_1). To accomplish this, we obtained bronchial biopsies from the patients before and after a 12–14 week course of treatment with omalizumab or placebo while maintaining their current therapy and evaluated mucosal IgE^+ cell numbers as a co-primary outcome measure. We then destabilised the patients over a further 6–8 week period, while maintaining omalizumab/placebo therapy, by staged reduction of existing anti-asthma therapy according to a protocol powered to detect changes in FEV_1, the primary outcome measure.

Study design, patients and laboratory methods

Study design

This was a randomised, placebo-controlled, double-blind, parallel-group, proof-of-concept study of 20 weeks' duration. Omalizumab and identical vehicle control manufactured to good clinical practice standards were kindly supplied by the manufacturers (Novartis, Basel, Switzerland). The trial was approved and monitored by Guy's Research Ethics Committee (REC Ref: 09/H0804/43) and the Medicines and Healthcare Products Regulatory Agency (CTA No: 14523/0219/001/0001) and registered on clinicaltrials.gov (reference NCT01113437). Eligible patients were uncontrolled, non-atopic asthmatics who provided written, informed consent recruited from the asthma clinics at Guy's and St. Thomas', the Royal Brompton and the Homerton University Hospitals in London, UK.

Asthma was defined as a history of relevant symptoms and documented. i) ⩾12% reversibility of FEV_1 in response to inhaled bronchodilator and/or ii) ⩾8% variability of the peak expiratory flow during a 24 h period or ⩾20% variability over a period of 1–2 weeks. Uncontrolled disease was defined as regular (at least 3 days per week) day- and night-time symptoms in the 3 months prior to screening despite regular step 3–5 asthma treatment according to the BTS guidelines. Non-atopic was defined as negative skin prick

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and/or *in vitro* IgE tests (Grade 0 or $\leqslant 0.35\ kU \cdot L^{-1}$, ImmunoCAP, Phadia, Uppsalla, Sweden) to the following local UK aeroallergens: mixed grass, mixed tree, mixed mould, cat, dog and house dust mite. The non-atopic status of these participants was further confirmed by full immuno-solid phase allergen chip (ISAC;Phadia) screening of their sera and bronchial biopsy homogenates (data presented elsewhere) [21]. Exclusion criteria are listed in the supplementary material.

The phases of the study protocol are summarised in figure 1.

Screening/baseline

After providing written, informed consent, during a baseline period of up to 4 weeks, patients were assessed for compliance with the inclusion/exclusion criteria and prepared for future visits. Existing anti-asthma medication was not changed but compliance encouraged.

First bronchoscopy and commencement of therapy

At a second visit patients completed a Juniper Asthma Control Questionnaire (ACQ) [27] and mini-Asthma Quality of Life Questionnaire (mini-AQLQ) [28], then underwent pre-bronchodilator spirometry (Minispir PC-based Spirometer, Winspiro Pro version 4.1.5 software; MIR, Rome, Italy) prior to the obtaining of 10 technically suitable bronchial mucosal biopsies from the right or left second- or third-generation bronchi at fibreoptic bronchoscopy using an Olympus bronchoscope model BF XT40 OES (Olympus, Tokyo, Japan). Patients then received their first subcutaneous injection of the trial medication (omalizumab or identical placebo, allocated by the hospital pharmacy using randomisation tables with the patient and attending physician blinded), the dosage and frequency of which (either 2 or 4 weekly) were determined as in standard clinical practice based on their initial body weight and serum total IgE concentration as described in the omalizumab summary of product characteristics. Where serum total IgE was below the lowest concentration in the summary of product characteristics dosing table we administered the lowest dosage in the table (75 mg every 4 weeks). Patients were observed for 2 h afterwards. At each subsequent dosing visit, patients were examined clinically and encouraged to comply with their usual medication.

Second bronchoscopy

Within a 2 week window between 12 and 14 weeks after commencement of omalizumab/placebo therapy (time A, figure 1), lung function was re-measured and repeat bronchial biopsies obtained as before.

Therapy reduction phase

Patients were instructed carefully how to use a Turbohaler device (AstraZeneca, London, UK) and asked, commencing the day following the second bronchoscopy, to discontinue all existing inhaled and oral anti-leukotriene or theophylline based anti-asthma medications and substitute them with regular budesonide/formoterol combination therapy (Symbicort 100/6 Turbohaler 2 puffs twice daily initially for 4 weeks and further reduced to 1 puff twice daily until the end of the study) with additional terbutaline (Bricanyl Turbohaler (AstraZeneca) 500 µg per puff) as required for immediate relief of symptoms. For patients taking regular additional oral prednisolone, an attempt was also made progressively to reduce the dosage according to a predetermined regimen depending on the dosage at entry to the study (supplementary table E1). Omalizumab/placebo therapy was continued for a total of 20 weeks while this new therapeutic regimen was pursued.

End of the study

At their penultimate visit, 20 weeks from commencement of omalizumab/placebo therapy (time B, figure 1), patients completed final ACQ and mini-AQLQ questionnaires then underwent repeat spirometry before

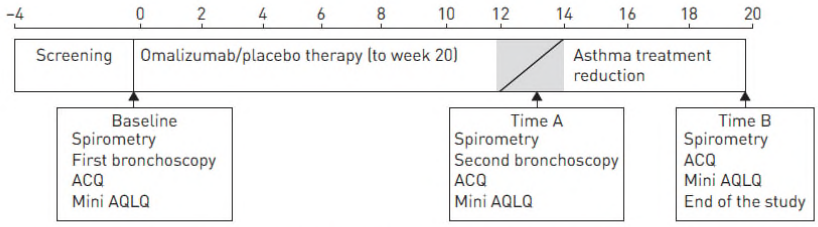

FIGURE 1 Clinical trial flow chart outlining interventions. Baseline: time from screening visit to first bronchoscopy and commencement of omalizumab/placebo (weeks −4 to 0); time A: time span during which the patients had a second bronchoscopy (weeks 12–14) after which therapy was reduced; time B: end of the trial 20 weeks from the first injection of omalizumab/placebo. ACQ: Asthma Control Questionnaire; mini-AQLQ: mini-Asthma Quality of Life Questionnaire.

being asked to resume their original anti-asthma therapy. A final visit was arranged 2 weeks later to check the patients' wellbeing and enquire about any adverse reactions.

At any time during the study, in the event of an asthma exacerbation, defined as a need for rescue oral corticosteroid medication for deterioration of symptoms and/or lung function, as agreed between the patient and the study physician, patients were treated with a 10-day course of prednisolone 30 mg·day^{-1} instituted by the study physician. Such patients left the study, resumed their regular anti-asthma medication and were followed up as necessary.

Immunofluorescence

Bronchial biopsies were processed and analysed using double or single immunofluorescence, single immunohistochemistry and confocal microscopy where appropriate according to our established protocols described in the supplementary material. Biopsy sections were analysed objectively by operators ignorant of their provenance.

Powering

In a previous therapy reduction study (CQAE397A2202) performed by Novartis in a similar patient group, the standard deviation of the differences in FEV_1 before and after reduction of inhaled corticosteroid was found to be 9.550. Using 20 subjects per group, this would give a 90% chance of detecting a 10% difference, with an alpha of 0.1 using a two-sided test. An investigator blinded interim analysis of differences in FEV_1, the primary outcome measure, following recruitment of 18 patients indicated a significant improvement in FEV_1 in the omalizumab, but not the placebo-treated group supporting our hypothesis, and so the study was terminated at this stage. Changes in numbers of IgE^+ cells, the co-primary outcome measure, were unprecedented and so could not be formally powered.

Statistical analysis

Baseline characteristics and demographic data were summarised using descriptive statistics. Changes in numerical variables at the beginning and end of the study as well as differences in changes between the omalizumab and placebo-treated groups were analysed by non-parametric statistics (Mann-Whitney U-test, Wilcoxon rank sum test). All tests were two sided and a p-value <0.05 was considered significant. The statistical software package used was GraphPad Prism version 5 (GraphPad Software, San Diego, CA, USA).

Results

Patients

The patients randomised to omalizumab or placebo were well matched in terms of distributions of age, sex, body mass index, serum total IgE concentrations, smoking history, lung function, asthma symptom scores and inhaled corticosteroid dosages (table 1). Inhaled corticosteroid dosages were reduced in both groups to 400 µg·day^{-1} beclometasone equivalent between 12–14 weeks and further to 200 µg·day^{-1} between 16 and 20 weeks. In the omalizumab *versus* placebo-treated groups, seven *versus* eight patients were taking long-acting β_2-agonist, four *versus* one were taking oral leukotriene receptor antagonist and two *versus* two were taking oral theophylline preparations. All these were stopped or substituted at time A (figure 1). One patient randomised to omalizumab and three to placebo were taking oral prednisolone at dosages of 15 mg·day^{-1} and 15, 10 and 5 mg·day^{-1} respectively which were reduced to 7.5 mg·day^{-1} and 7.5, 5 and 0 mg·day^{-1} respectively according to the predetermined regimen (supplementary table E1). Of 18 patients randomised, 15 completed the study. One patient allocated to placebo and two to omalizumab withdrew prematurely (see Discussion section).

Airways inflammation

The median numbers of $tryptase^+$ mast cells, $CD20^+$ B cells, $CD138^+$ plasma cells, $CD38^+$ plasmablasts, total IgE^+ cells, IgE^+/$tryptase^+$ mast cells, IgE^+/$CD20^+$ B cells, IgE^+/$CD138^+$ plasma cells, and Barkans Moqbel Kay-13 ($BMK\text{-}13^+$) eosinophils per unit area of the bronchial biopsy sections just prior to commencement of omalizumab and at time A (after 12–14 weeks of omalizumab or placebo therapy with no alteration of existing medications, see figure 1) are shown in table 2, while selected changes are depicted graphically in figure 2. We observed a significant reduction in the median total IgE^+ cells ($p<0.001$), the co-primary outcome measure, in the bronchial mucosa in the patients treated with omalizumab but not placebo. No significant changes were observed in median numbers of any of the other cells analysed following omalizumab or placebo therapy. Very few of the B cells and plasma cells showed detectable IgE immunoreactivity as expected (table 2), so it was impracticable to evaluate changes. Clusters of $CD38^+$ plasmablasts and $CD20^+$ B cells were detected in approximately one in every 10 sections of the mucosa (figure 2f) but again these were insufficient in number to quantify possible changes.

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TABLE 1 Baseline demographics and clinical characteristics of non-atopic asthmatics randomised to omalizumab or placebo therapy and absolute changes from baseline at times A and B

Characteristics and outcomes	Baseline	Time A	Time B
Age years			
Omalizumab	47 (22–66)		
Placebo	53.50 (25–59)		
Male/female n			
Omalizumab	5/3		
Placebo	3/5		
Weight kg			
Omalizumab	84 (55–133)		
Placebo	87.50 (61–112)		
Height cm			
Omalizumab	172 (155–178)		
Placebo	166 (152–177)		
Never-smokers n (of 8 total)			
Omalizumab	3		
Placebo	4		
Serum total IgE IU·mL^{-1}			
Omalizumab	75 (8–264)		
Placebo	49 (2–284)		
FEV_1 L			
Omalizumab	2.44 (1.81–3.43)	0.225 (−0.32–1.24)	0.26 (0.03–0.67)[#]
Placebo	2.34 (1.16–3.66)	0.005 (−0.30–0.45)	−0.06 (−0.63–0.39)
FEV_1% predicted			
Omalizumab	75 (50–98)	6 (−13–36)	11 (1–19)[¶]
Placebo	80 (47–114)	−4 (−11–14)	−2 (−23–15)
ACQ score			
Omalizumab	2.28 (1.43–3.43)	−0.50 (−1.85–0.72)	−0.71 (−1.14–0.14)
Placebo	2.42 (0.71–3.28)	−0.35 (−1.00–0.57)	−0.28 (−1.71–1.14)
Mini-AQLQ score			
Omalizumab	4.33 (2.53–5.27)	0.18 (−1.00–2.26)	0.46 (−0.40–2.06)
Placebo	4.60 (3.73–5.80)	0.37 (−1.34–1.87)	0.67 (−1.60–2.30)
ICS BDP equivalent µg·day^{-1}			
Omalizumab	2000 (800–4000)	No change from baseline	Reduced to 200 µg BDP equivalent per day
Placebo	1800 (500–2000)	No change from baseline	Reduced to 200 µg BDP equivalent per day

Data are presented as median (range). FEV_1: forced expiratory volume in 1 s; ACQ: Juniper Asthma Control Questionnaire; mini-AQLQ: Juniper mini-Asthma Quality of Life Questionnaire; ICS: inhaled corticosteroids; BDP: beclometasone dipropionate. [#]: p=0.04, [¶]: p=0.015 compared with baseline (Mann-Whitney U-test).

Lung function and asthma symptom scores

The primary outcome measure in this study was the change in median FEV_1 in the omalizumab compared with the placebo treated patients during the study (between baseline and time B in figure 1). The median absolute and % predicted FEV_1 improved during this period in the omalizumab treated patients, despite substantial reduction of existing therapy, but deteriorated in the placebo-treated patients as anticipated (median (semi-interquartile range) change 0.26 (0.07–0.64) *versus* −0.06 (−0.14–0.27) L; p=0.04; 11 (2–18) *versus* −2.0 (−11–1.5) % predicted; p=0.015; see figure 3). As an exploratory measure, we also recorded asthma symptom scores. As shown in table 1, the median ACQ score between baseline and 20 weeks (time B) improved by what is regarded as a clinically meaningful degree in the patients randomised to omalizumab but not placebo despite the staged reductions in existing therapy, although the differences between the groups did not attain statistical significance. In contrast, median mini-AQLQ scores improved to a similar extent in both groups.

Discussion

We conceived this study to challenge, in the light of current knowledge, the tacit assumption that omalizumab therapy is of no clinical benefit in conventionally defined, non-atopic asthmatics. In lieu of a large, long-term, conventional clinical trial in the first instance, which would have been difficult to fund given the paucity of supportive evidence, we elected to provide proof of concept that omalizumab therapy of these patients reduces IgE expression and IgE sensitisation of target cells within the bronchial mucosa while exerting a favourable effect on lung function in the short term, as assessed by changes in FEV_1. We

TABLE 2 Bronchial mucosal inflammatory cells: absolute counts per mm^2 at baseline and absolute changes at time A following treatment with omalizumab or placebo

Cell type	Absolute numbers per mm^2 at baseline	Difference between baseline and time A
Tryptase$^+$ mast cell		
Omalizumab	7.22 (0–124.77)	10.88 (−61.64–57.70)
Placebo	29.92 (4.32–75.64)	3.68 (−14.66–13.08)
CD20$^+$ B cell		
Omalizumab	1.48 (0.00–9.67)	0.00 (−8.25–4.80)
Placebo	2.10 (0.29–9.35)	−0.15 (−1.32–6.39)
CD138$^+$ plasma cell		
Omalizumab	0.00 (0.00–36.63)	0.00 (−4.42–3.47)
Placebo	1.68 (0.00–6.80)	−0.24 (−1.93–1.13)
CD38$^+$ plasmablast		
Omalizumab	3.91 (0.00–14.57)	3.07 (−3.83–69.72)
Placebo	0.61(0.00–39.39)	−0.21 (−28.39–8.67)
IgE$^+$ cell		
Omalizumab	12.38 (1.22–175.90)	−5.44 (−109.89–−1.30)**
Placebo	16.45 (2.86–60.92)	4.98 (−17.08–17.81)
IgE$^+$ mast cell		
Omalizumab	9.19 (0.00–124.46)	−1.89 (−99.94–33.31)
Placebo	26.16 (4.32–50.23)	7.88 (−17.58–24.08)
IgE$^+$ B cell		
Omalizumab	0.00 (0.00–0.28)	0.00 (0.00–0.32)
Placebo	0.00 (0.00–0.00)	0.00 (0.00–0.00)
IgE$^+$ plasma cell		
Omalizumab	0.00 (0.00–5.98)	0.00 (−5.41–0.00)
Placebo	0.00 (0.00–0.46)	0.00 (−0.46–0.00)
BMK-13$^+$ eosinophil		
Omalizumab	9.03 (2.92–17.20)	−0.76 (−14.40–4.47)
Placebo	4.26 (0.31–21.36)	0.81 (−9.65–7.57)

Data are shown as the median and range. BMK-13: Barkans Moqbel Kay-13. **: p<0.01.

obtained bronchial biopsies before and after 12–14 weeks of treatment with omalizumab or placebo while maintaining existing therapy, reasoning that any differences in mucosal cell populations between the active and placebo cohorts observed under these conditions would be attributable to omalizumab. We set changes in mucosal IgE$^+$ cells as a co-primary outcome measure, although we were unable to power the study to detect such changes *a priori* because of lack of any pre-existing data. To obtain proof of concept that omalizumab can improve lung function, we then destabilised the patients by staged reduction of their therapy according to a protocol previously validated by Novartis and powered to detect changes in FEV_1. Although we failed to recruit 40 patients as suggested by the original power calculation (recruitment of patients with severe asthma willing to undergo two bronchoscopic procedures followed by supervised reduction of their existing therapy and attend 10 study visits over a period of 6 months was a considerable challenge), we were able to demonstrate with considerably fewer patients a clear and statistically significant effect of omalizumab but not placebo in improving FEV_1 within a time frame used to gauge responsiveness clinically. In fact, all of the patients treated with omalizumab improved their FEV_1 over the 20 week period of the study, despite substantial reduction of their existing therapy. We also explored changes in quality of life and, although the study was not powered to examine these it revealed further intriguing data, particularly an improvement in ACQ score, which is arguably more reflective of short-term asthma stability than mini-AQLQ, which may improve in response to increased surveillance in the context of a clinical trial. These data are congruent with a previous, ground breaking, placebo-controlled proof-of-concept clinical trial by GARCIA *et al.* [29] examining the clinical effects of omalizumab in non-atopic asthmatics, who were also able to demonstrate improvement of FEV_1 in their patients and a trend towards improvement in global evaluation of clinical effectiveness and asthma exacerbation rate.

Two patients randomised to omalizumab therapy withdrew, the first following an exacerbation just after commencing omalizumab and prior to any change in therapy. The second patient elected to withdraw during the therapy reduction phase despite lack of any objective evidence of deterioration. The data from this patient's bronchial biopsies, but none of the clinical data were included in the final analysis. One patient assigned to placebo withdrew immediately following screening. Although in larger studies

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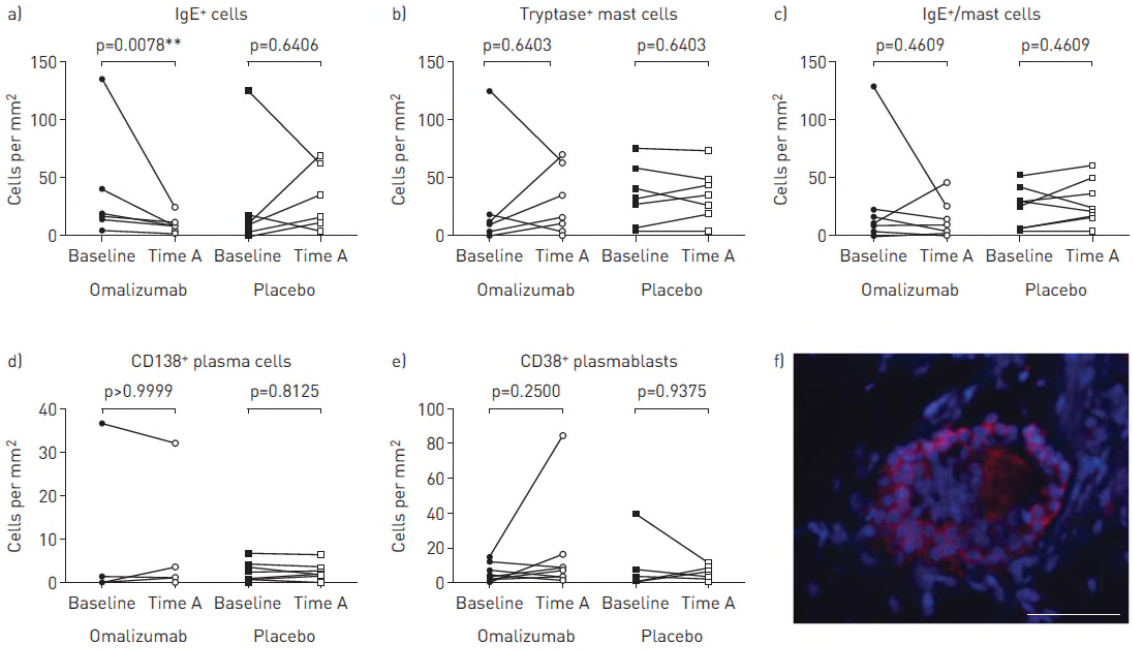

FIGURE 2 Effects of omalizumab and placebo treatment on numbers of bronchial mucosal a) total IgE$^+$ cells; b) tryptase$^+$ mast cells; c) IgE$^+$/tryptase$^+$ mast cells; d) CD138$^+$ plasma cells; e) CD38$^+$ plasmablasts. Wilcoxon rank sum test used for comparison. f) Germinal centre-like follicle of CD38$^+$ plasmablasts (red) counterstained with nucleoprotein (blue). Scale bar=50 µm

withdrawal rates may reflect the efficacy of a novel therapy, this is clearly not applicable in the present study. One patient assigned to omalizumab and three to placebo were taking oral corticosteroids. Since we hypothesised that omalizumab reduces IgE expression in the target organ of non-atopic asthmatics whose existing therapy is maintained, and that omalizumab therapy preserves/improves FEV_1 in the face of significant therapy reduction, it is unlikely that our conclusions have been influenced by this.

Having demonstrated that IgE is increased in the bronchial mucosa of non-atopic asthmatics [21], we here additionally show that omalizumab therapy substantially reduces the numbers of cells exhibiting associated IgE, consistent with our hypothesis. This presumably reflects sequestering by omalizumab of free IgE

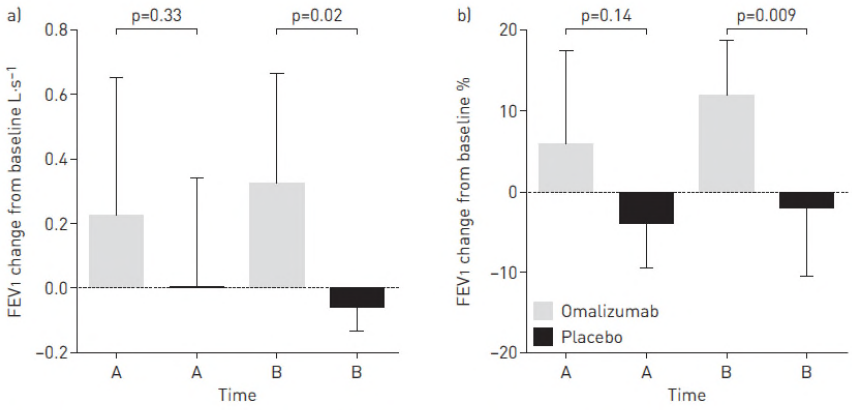

FIGURE 3 Comparison of effect of treatment with omalizumab and placebo on changes in absolute and % predicted forced expiratory volume in 1 s (FEV_1) between baseline and times A and B. Bars represent the median and interquartile range. Mann–Whitney U-Test used for comparison.

within the bronchial mucosa, resulting in down regulation of its receptors on local target cells [30–32]. Similarly, in a study of mild atopic asthmatics [33], treatment with omalizumab reduced numbers of bronchial mucosal FcεRI$^+$ and IgE$^+$ cells. Although this is the only clearly described anti-inflammatory activity of omalizumab, there is little understanding of its functional consequences for asthma severity and control, especially in a setting of non-atopic disease. Indeed, in the absence of a clear correlation between down regulation and IgE$^+$ cells and clinical efficacy in the omalizumab-treated patients in our study, it is possible to hypothesise that this effect is epiphenomenal and not relevant to amelioration of asthma. It is well established that the clinical effectiveness of omalizumab varies in individual patients in a manner not predicted by their baseline, circulating IgE concentrations [34], suggesting that responsiveness does not simply reflect overall IgE production (at least in the circulation). In addition, CHANEZ *et al.* [35] showed that, while in a group of severe atopic asthmatics omalizumab therapy reduced expression of FcεRI on blood basophils and plasmacytoid dendritic cells (a finding also confirmed in the study by GARCIA *et al.* [29]), there was no correlation with clinical responsiveness.

To further explore possible functional consequences of omalizumab therapy we looked for changes in inflammatory cell numbers in the bronchial mucosa (such changes cannot prove cause and effect but may provide mechanistic clues). In the event, we observed none. The study may not have been sufficiently powered to detect such changes. A further caveat is the issue of cell clusters. It was with great interest that we noted clusters of CD38$^+$ plasmablasts and CD20$^+$ B cells in approximately one in 10 sections of the bronchial mucosa, consistent with our previous observation that these cells switch locally to IgE synthesis [18]: this would likely be accompanied by multiple cellular divisions [36]. It is technically challenging to enumerate changes in numbers of cells distributed as discrete clusters. Even assuming, however, that omalizumab does also inhibit local B cell differentiation and IgE synthesis in the bronchial mucosa, consistent with its known effects on IgE expressing B cells and antigen-presenting cells [37, 38], it is again not yet clear if and why this may be important in asthma.

Designed as it was to provide proof of concept, the present study has limitations. The study was neither designed nor powered to detect influences of omalizumab therapy on "conventional" clinical outcomes; in particular, changes in quality of life or disease exacerbation rate. We were unable, as mentioned, to power the study for the proposed histopathological end points because of paucity of prior, relevant data and because of the difficulty of enumerating cells in clusters in very small samples of the bronchial mucosa.

Notwithstanding these observations, our findings and those of others referred to herein arguably merit closer examination of the therapeutic worth of anti-IgE therapies in non-atopic asthma in larger studies with conventional clinical outcomes, potentially addressing a large therapeutic need.

The greater challenge for the future will be to uncover further mechanisms whereby IgE can regulate the severity and stability of asthma, enabling more substantial appraisal of the likelihood that any given individual will respond clinically to anti-IgE therapy using criteria which extend beyond the boundaries of conventional atopic status. This is a key theme of our ongoing research.

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Appendix 3.2: “Allergen-specific IgE is not detectable in the bronchial mucosa of non-atopic asthmatic patients”

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Allergen-specific IgE is not detectable in the bronchial mucosa of nonatopic asthmatic patients

To the Editor:

Activation by aeroallergen-specific IgE of bronchial mucosal mast cells in clinically sensitized atopic subjects is an acknowledged exacerbating (some speculate causative) stimulus for asthma, although the existence of the disease in nonatopic subjects, who are conventionally defined as those with absence of allergen-specific IgE, as determined by using skin prick and *in vitro* tests, begs the question of whether allergens and IgE play an indispensable role in asthma pathogenesis. We have previously shown that nonatopic asthmatic patients, like atopic patients, display the entire molecular machinery to support B-cell switching in the bronchial mucosa[1] and that this does indeed occur,[2,3] with indirect evidence of increased local IgE synthesis.[4] Epidemiologically, increased IgE production is a major risk factor for asthma, regardless of atopic status.[5]

One possible explanation for this paradox is that IgE is manufactured in the bronchial mucosa of nonatopic asthmatic patients but remains confined there and bound to cells bearing its receptors. To address this, we hypothesized that local IgE synthesis in the bronchial mucosa of both atopic and nonatopic asthmatic patients results in greater local total IgE concentrations than in serum and, in the case of nonatopic asthmatic patients, production of allergen-specific IgE that is sequestered in the mucosa and therefore not detectable in the periphery. We determined the concentrations and allergen specificities of IgE in the blood and bronchial mucosa of a group of atopic and nonatopic asthmatic patients and nonatopic control subjects, not only to a panel of allergens conventionally used for skin prick testing in the United Kingdom but also to an extended panel of more than 100 known allergen components.

There were 10 subjects in each study group (Table I). All provided written informed consent to participate in the study, which was approved by a local research ethics committee. Ten bronchial mucosal biopsy specimens were collected from the right or left, second- or third-generation bronchi at fiberoptic bronchoscopy. Biopsy specimens were weighed, then snap-frozen and homogenized (see the Methods section in this article's Online Repository at www.jacionline.org). Serum was collected on the same day. Samples were stored at −80°C until analysis. Analysis was generally performed on pooled triplicate biopsy extracts; however, all 10 biopsy specimens were analyzed individually in one of the subjects to assess between-specimen variability (see the Methods section in this article's Online Repository at www.jacionline.org). Total and allergen-specific IgE concentrations in aliquots of serum and mucosal homogenates were measured by using commercial assays (Phadia ImmunoCAP-100 and ImmunoCAP ISAC, respectively; Phadia, Uppsala, Sweden), according to the manufacturer's instructions (see the Methods section in this article's Online Repository at www.jacionline.org).

By using these techniques, IgE was detectable in the serum and bronchial mucosa of all study subjects (see Fig E1 in this article's Online Repository at www.jacionline.org). The median total IgE concentrations in mucosal homogenates were significantly increased in both the atopic and nonatopic asthmatic patients compared with those seen in control subjects, even allowing for variability of IgE concentrations between different mucosal sites (Fig 1 and see Fig E2 in this article's Online Repository at www.jacionline.org). Interestingly, this was not reflected in sera, in which only the median total IgE concentration in atopic asthmatic patients was significantly greater than in control subjects, although with a similar trend in the nonatopic asthmatic patients. By using the ImmunoCAP ISAC microarray, some allergen component–specific IgE species were detectable in sera and mucosal homogenates from the atopic asthmatic patients but not the nonatopic asthmatic patients and control subjects. Where detectable, the relative concentrations of a minority of these species, expressed as percentages of total IgE (see Figs E3-E5 in this article's Online Repository at www.jacionline.org) or absolute concentrations (see Tables E1-E3 in this article's Online Repository at www.jacionline.org), were higher in the bronchial mucosa than in the serum, although most were lower. To investigate the possible heterogeneity of distribution of allergen component–specific IgE in the bronchial mucosa, we compared concentrations in 9 geographically separate biopsy specimens from a single atopic asthmatic patient (see Table E4 in this article's Online Repository at www.jacionline.org). Component-specific IgE was detectable in 4 of the 9 biopsy specimens, typically when the serum concentration of the same species was particularly increased.

So far as we are aware, this is the first study in which total and allergen-specific IgE levels have been measured successfully in the bronchial mucosa. The data are consistent with the hypothesis that IgE synthesis, sequestration, or both are ongoing in the bronchial mucosa of both nonatopic and atopic asthmatic patients, but not that this IgE is directed against common, local aeroallergens in nonatopic asthma. Although the data do not distinguish local *de novo* IgE synthesis from increased IgE sequestration by binding to IgE receptor–bearing cells, we have been able to identify $IgE^+/CD138^+$ plasma cells, as well as IgE^+/tryptase-positive mast cells, after preliminary sequential immunofluorescence immunohistochemical analysis of sections of further biopsy specimens from the same nonatopic asthmatic

TABLE I. Demographics and lung function of study subjects and corticosteroid therapy of asthmatic patients

Subject group	Age (y)	Sex (F/M)	FEV_1 (% predicted)	Inhaled corticosteroid dosage (g/d BDP equivalent)
Nonatopic control subjects	23 (21-36)	3/7	104.5 (94-128)	
Nonatopic asthmatic patients	54 (24-70)*	4/6	67.5 (40-101)†	1800 (640-4000)
Atopic asthmatic patients	30.50 (24-66)*	4/6	95 (64-130)‡	150 (0-1600)§

Age, FEV_1 percent predicted, and inhaled corticosteroid dosages are expressed as medians (ranges). Three nonatopic asthmatic patients were taking regular oral prednisolone in addition to inhaled corticosteroids.
BDP, Beclomethasone dipropionate; *F*, female; *M*, male.
*$P \leq .01$ and †$P \leq .005$ versus control subjects and ‡$P = .02$ and §$P = .002$ versus nonatopic asthmatic patients (Mann-Whitney *U* test).

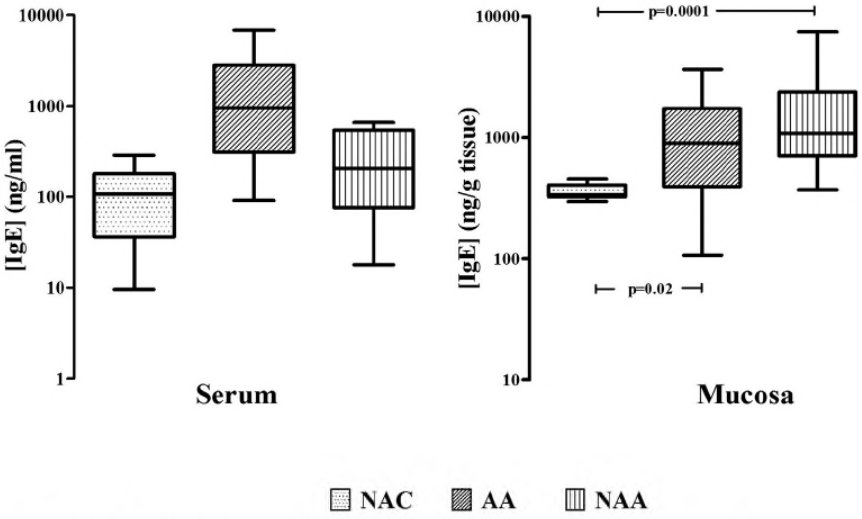

FIG 1. Box and whisker plots summarizing total IgE concentrations in the serum *(left)* and bronchial mucosa *(right)* of nonatopic control subjects *(NAC)*, atopic asthmatic patients *(AA)*, and nonatopic asthmatic patients (*NAA*); Mann-Whitney *U* test.

patients (Fig 2). Because plasma cells do not express IgE Fc receptors, this expression is likely to reflect endogenous IgE synthesis. Of course, the data do not indicate the contribution of this synthesis to total IgE concentrations or provide an impression of the uniformity or otherwise of these findings over the vast surface area of the entire bronchial mucosa. However, we would speculate that examination of limited numbers of biopsy specimens, which we are constrained to do by practical and ethical considerations, considerably underestimates the total numbers of IgE-producing plasma cells present in the entire bronchial mucosa. In addition, although our data do not clearly support the hypothesis that the bronchial mucosa is a site of abundant synthesis of allergen-specific IgE in atopic subjects, they clearly suggest that IgE synthesis can occur at this site.

Although the variation in total IgE concentrations between biopsy specimens was no more than 2-fold within subjects in the present study, our data suggest that there might be regional differences in local and allergen-specific IgE concentrations brought about, for example, by local clonal expansion of B cells, variations in allergen exposure, and variable sequestering by IgE receptor–bearing cells. There could also theoretically be temporal differences, for example reflecting seasonal allergen exposure or changes in global IgE production with age, and further variations reflecting exposure to antiasthma therapy. Bronchial biopsy specimens sample only a miniscule proportion of the total area of the lower airways mucosa, and biopsy specimens from some of the atopic subjects contained considerably more allergen-specific IgE than serum (see Tables E1-E3), which is consistent with the possibility of local production. It is also possible that allergen-specific IgE synthesis is more prevalent in the upper airways mucosa, where allergens are more likely to be retained.[6] We cannot completely exclude the possibility that nonatopic asthmatic patients produce allergen-specific IgE locally but in amounts less than the threshold of our detection methods, although this seems very unlikely because it is self-evident that our technique is capable of detecting such IgE in mucosal homogenates and would have done so had it been present in the nonatopic asthmatic patients in similar quantities.

Finally, and importantly, our data suggest the possibility that increased bronchial mucosal IgE production in nonatopic asthmatic patients is directed against targets other than allergens, including possible "autoallergens," a scenario for which there is ample and increasing precedent,[7-9] or that there are allergen-independent roles for IgE in the pathophysiology of asthma. It is now vitally important that the specificities and functional activities of these antibodies be fully investigated.

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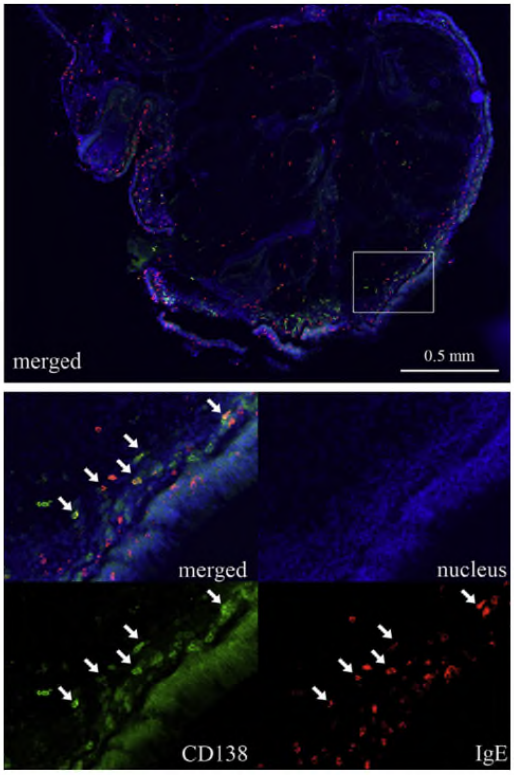

FIG 2. Immunofluorescence image of a bronchial biopsy specimen from a nonatopic asthmatic patient stained for plasma cells (CD138; *green*), IgE *(red)*, and nucleus *(blue)*. *Top,* View of the whole biopsy specimen showing area of magnification. *Bottom,* Magnified view with a merged image and individual stains. *Arrows* indicate cells with dual staining (plasma cells expressing IgE).

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Defining risk factors and presentations of allergic reactions to platelet transfusion

To the Editor:

Allergic transfusion reactions (ATRs) are a frequent complication of blood transfusion, occurring in at least 2% of platelet transfusions.[1,2] The mechanisms of most ATRs are not understood. Antibodies to plasma proteins such as IgA,[3,4] haptoglobin,[5] and C4[6] in blood transfusion recipients are reported but rarely identified. Furthermore, individuals react sporadically to transfusions, which is inconsistent with a recipient having hypersensitivity to a ubiquitous plasma protein.

We hypothesized that understanding the epidemiology and clinical presentation of ATRs will elucidate the underlying mechanism and guide strategies to prevent ATRs. We developed a cohort of platelet donors and the platelet transfusion recipients who received their donated products. The goals of this study were to (1) identify risk factors for ATRs, using platelet transfusion as a paradigm, and (2) define the clinical and biochemical presentation of ATRs.

Appendix 4: Comments from the statistician

PhD thesis

RF

Reid, Fiona
Today, 16:19
Chellappan Pillai, Prathap

Reply all

Inbox

You replied on 31/10/2017 18:36.

Action Items

Dear Prathap,

As discussed, I set out below my thoughts on the analysis of omalizumab vs placebo in Fig 3.1, as requested by your PhD examiners.

King regards,

Fiona Reid

Prathap Pillai

PhD Thesis – Examiners' comments

Comment 11. (Analysis underpinning Fig 3.1)

As I understand the study and its protocol, I believe the comparison of outcomes (including FEV1) between the omalizumab group and the placebo group at 12-14 weeks (time A) and at 20 weeks (time B) are included in the study's aims. The comparison of FEV1 at 12 weeks has been adjusted for baseline FEV1 by using change scores from baseline; similarly the comparison at 20 weeks. This approach is less optimal than an analysis of covariance, where baseline scores are the covariate, but is an acceptable alternative where non-parametric analysis has been selected. Adjusting for baseline FEV1 is important to remove the natural 'variation' in FEV1 between different patients who may have very different starting points.

A comparison of FEV1 between 12 weeks and 20 weeks has been proposed in the PhD comments. I am not 100% sure what this means, but one interpretation would be a comparison of the omalizumab group and the placebo group at 20 weeks, adjusted for the 12 week scores. In this case the adjustment would not just be for the natural baseline variation, but also for the differences achieved so far at 12 weeks, which I don't think makes sense.

I believe the analysis as currently presented is appropriate and reflects the study protocol.

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Please note that my usual work days are Monday to Thursday